PURIFICATION AND PROPERTIES OF PECTINESTERASES FROM MARSH WHITE GRAPEFRUIT PULP

THOMAS A. SEYMOUR

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1990

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Drs. Maurice R. Marshall and Louise Wicker, the co-chairmen of my graduate supervisory committee for their scientific support and encouragement. I would like to extend my gratitude to Dr James F. Preston for his special assistance and suggestions to improve the depth of this project. My special thanks are extended to Drs. Ken M. Dunn and James A. Lindsay for their assistance, advice and support. Finally, I wish to thank my wife, Haejung, for her invaluable support and encouragement.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS ii

LIST OF TABLES v

LIST OF FIGURES vi

ABSTRACT ix

INTRODUCTION 1

LITERATURE REVIEW 5

Literature on Pectinesterase 5

Structure-stability Relationship in Proteins 25

MATERIALS AND METHODS 31

Preparation of Crude Extracts 31

Enzyme Assays 33

Protein Assays 35

Pectin Analysis 36

Column Chromatography 39

Molecular Characterization of Purified Pectinesterases 42

Kinetics and Stability 51

Carbohydrate Composition and Function 54

RESULTS AND DISCUSSION 60

Purification 60

Molecular Characterization.... 76
Kinetics and Stability..... 95
Composition and Function of
Carbohydrate in Pectinesterases.... 112
SUMMARY.... 132
REFERENCES.... 136
BIOGRAPHICAL SKETCH.... 148

LIST OF TABLES

Table Page

1. Extraction and purification of TL PE 66
2. Extraction and purification of TS PE 74
3. Amino acid composition of purified grapefruit pectinesterases 82
4. Amino acid composition of purified orange and tomato pectinesterases 83
5. Average hydrophobicity and thermostability of pectinesterases 84
6. Properties of purified pectinesterases 90

LIST OF FIGURES

Figure Page

1. Purification of TL PE: Elution pattern from CM-Sephadex column chromatography of crude PE 62

2. Purification of TL PE: Isocratic elution pattern from Sephacryl-200 gel filtration column chromatography of CM-Sephadex fractions 64

3. Purification of TS PE: Gradient elution pattern from CM-Sephadex column chromatography of heat-treated crude PE 69

4. Purification of TS PE: Gradient elution pattern from CM-BioGel A column chromatography 71

5. Purification of TS PE: Isocratic elution pattern from G-75 gel filtration column chromatography 73

6. Purified TS PE and TL PE on 12% SDS-PAGE mini-gel stained with Coomassie blue/amido black 78

7. Determination of the native MW of TS PE and TL PE by gel filtration on HPLC Superose-6 80

8. Analysis of cyanogen bromide cleavage products of TL and TS PE by SDS-PAGE 88

9. Titer of anti-TL PE chicken antibodies in eggs 92

10. Analysis of antigenic properties of purified TS and TL PE by competitive ELISA 94

11. Lineweaver-Burk plot of the activity of TS and TL PE at various concentrations of Sunkist pectin 97

12. The effect of pH on reaction rate of TS and TL PE on Sunkist pectin 99

13. Effect of degree of esterification on the reaction rate of TS and TL PE 102

14. Storage stability of TL PE in grapefruit juice .. 104

15. Storage stability of TS PE in grapefruit juice .. 106

16. Heat stability of pectinesterases in grapefruit juice 109

17. Thermal inactivation rate of TS PE in grapefruit juice at 75°,80° and 85°C 111

18. Effect of proteases on activity of pectinesterases 114

19. Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides of the total hydrolysate of TS PE 118

20. Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides from the total hydrolysate of TS PE 120

21 Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides released from TS PE following reductive β-elimination and subsequent acid hydrolysis 122

22. β-elimination reaction of TS PE in alkali solution 125

23. Heat stability of native and periodate-treated TS PE 128

24. Stability of native and periodate-treated TS PE 131

ABBREVIATIONS

AGA	-Anhydrogalacturonic acid
CM	-Carboxymethyl
DE	-Degree of esterification
DP	-Degree of polymerization
ELISA	-Enzyme-linked immunosorbent assay
GC/MS	-Gas chromatography/mass spectroscopy
HMW PE	-High molecular weight pectinesterase
HPLC	-High performance liquid chromatography
MW	-Molecular weight
PBS	-Phosphate-buffered saline
PVDF	-Polyvinylidene difluoride
PE	-Pectinesterase
PG	-Polygalacturonase
R_f	-Relative mobility
SDS-PAGE	-Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TL PE	-Thermolabile pectinesterase
TFMSA	-Trifluoromethanesulfonic acid
Tris	-Tris (trihydroxymethyl) aminomethane
TS PE	-Thermostable pectinesterase

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

PURIFICATION AND PROPERTIES FROM PECTINESTERASES OF MARSH WHITE GRAPEFRUIT PULP.

By

Thomas A. Seymour

May, 1990

Chairman: Maurice R. Marshall
Major Department: Food Science and Human Nutrition

Pectinesterase (PE) is an enzyme of significant concern to the citrus industry. It has been definitively established as the causative agent for clarification of citrus juice and gelation of frozen concentrates. This enzyme is present in multiple forms in citrus fruits. Thermostable forms of citrus PE have been described. Although representing a minor fraction of the total PE activity, these account for the severe time/temperature processing treatment required to inactivate PE. Currently inactivation of citrus PE is accomplished by treatment in the Thermally Accelerated Short Time Evaporator (TASTE) during concentration or by pasteurization at 90°C for 1 min. The high processing temperature causes a cooked off-flavor in the juice.

This study was undertaken to characterize novel forms of citrus PE and to determine a molecular basis for the differences in thermostability among the multiple forms of citrus PE. The approach taken was to analyze and compare the structures of thermostable and thermolabile forms of PE purified from Marsh white grapefruit pulp.

Two forms of PE were purified from Marsh white grapefruit pulp by ion exchange and gel filtration chromatography. They were found to be significantly different in thermostability; therefore, they were referred to as thermolabile (TL PE) and thermostable (TS PE) pectinesterases. Thermolabile PE accounted for the majority of PE activity in crude extracts (90%). The native molecular weights of TL PE and TS PE were estimated to be 36 and 51 kD, respectively. Both enzymes were composed of a single polypeptide chain. Carbohydrate content was estimated to be 2% for TL PE and 14 2% for TS PE. Analysis of amino acid content showed significant differences between TL PE and TS PE. Partial removal of the carbohydrate moiety of TS PE decreased stability significantly. The observed difference in stability of these enzymes therefore is at least partly due to carbohydrate content. Differences in primary structure may also contribute to enhanced stability of TS PE.

INTRODUCTION

Florida citrus is one of the largest agricultural commodities in the nation. About one-third of the world's orange supply and more than one-half of the world's grapefruit supply are produced in Florida. Approximately 93% of orange and 66% of grapefruit are processed for an added value of about $2.5 billion (Florida Crop and Livestock Reporting Service, 1985).

Pectinesterase (PE) is of great concern to the citrus industry since it has been definitively established as the causative agent for clarification of citrus juices and gelation of concentrates (Josyln and Pilnik, 1961; Krop, 1974). Incomplete inactivation of pectinesterase during processing can lead to serious quality defects in citrus juice and other citrus beverage containing drinks. This enzyme, present in abundance, exists in multiple forms in citrus fruits, some of which possess exceptional thermostability characteristics (Evans and McHale, 1978; Korner et al., 1980; Versteeg et al., 1978; Versteeg, 1979). Although representing 5-30% of the total activity in citrus fruits (Rombouts et al., 1982, Versteeg, 1979,), this enzyme(s) is the most active form in chilled juice (Versteeg et al., 1980), and accounts for the severe time/temperature profile needed to inactivate pectinesterase in citrus juice (Versteeg, 1979; Versteeg et al., 1980). It has been suggested that thermostable pectinesterase(s) is largely responsible for cloud loss of underpasteurized citrus juices (Versteeg et al., 1980).

Currently, citrus pectinesterase activity is controlled by one of three methods: by inactivation concomitantly with the concentration process in the Thermally Accelerated Short Time Evaporator (TASTE), by pasteurization at 90°C for one minute, or by inhibition by frozen storage at -20°C (Joslyn and Pilnik, 1961). The high temperature necessary for inactivation results in cooked off-flavors (Kew and Veldhuis, 1961). The recent development of lower temperature methods to concentrate juice (freeze concentration, membrane concentration, etc.), in combination with an increasing preference by consumers for lightly processed juice over pasteurized shelf-stable juice, suggests that development of low-temperature processing methods would increase substantially the flavor quality and economic value of citrus juice.

Although a major problem in citrus, the thermostability of citrus PEs could be a distinct advantage to the beverage industry for use in immobilized bioreactors and in other applications. Clarification of apple juice (as well as beer and wine) with immobilized thermostable enzymes should offer improved process control and cost savings over the currently used batch operations. Viscosity reduction of citrus pulp wash with immobilized thermostable pectolytic enzymes offers similar process control advantages. Therefore, thermostable citrus PE could potentially become a valuable by-product for the beverage industry.

Processing factors which influence the time/temperature requirement for the inactivation of PE have been well characterized. Kinetics and stability of three forms of PE in navel oranges have been determined; however, the molecular basis for the observed differences in these properties among the various forms is unknown. In order to enhance the prospects for the development of an alternative strategy for in vivo or in vitro control of PE, information is needed regarding the structural features which account for the kinetic and stability differences. The main objective of this research project was to identify structures which contribute to the extreme heat stability of the thermostable PE present in grapefruit pulp. The approach taken to achieve this goal was to compare the structures of the thermostable PE with that of a less thermostable form from the same source. Successful analyses, of course, depended on purified preparations of each enzyme to ensure definitive structural comparisons.

The specific objectives of this work were:

1) purification of pectinesterase from Marsh white grapefruit pulp and separation into component forms;

2) characterization of the enzymes with respect to molecular properties, kinetics, and stability;

3) determination and comparison of the structural features which account for differences in stability.

LITERATURE REVIEW

Literature on Pectinesterase

General

As this dissertation is concerned with citrus pectinesterase (PE), the literature on plant PE is reviewed in this chapter. Pectinesterases from other sources are described only briefly.

The substrate of PE, the pectic polysaccharides, are a major constituent of the plant middle lamella and primary cell wall. The basic structural elements of pectic polysaccharides have been described in many reviews (Aspinall, 1980; Darvill et al., 1980; Fogarty and Ward, 1974; Pilnik, 1981; Selvendran, 1983; Towle and Christensen, 1973; Voragen and Pilnik, 1970). Pectic polysaccharides are composed of a rhamno-galacturonan backbone in which partially methyoxylated (1-4)-α-D-galacturonan chains are interrupted at intervals by (1-2)-α-L-rhamnose units. Highly methoxylated galacturonan chains are referred to as pectin, while low methoxyl or methoxyl-free chains are referred to as pectinic or pectic acid, respectively. Other constituent sugars, which are attached in side chains to L-rhamnose, include predominantly arabinose, galactose, and xylose. By specific degradation of the galacturonan backbone of purified pectins from various sources, essentially pure, degraded galacturonan fractions have been obtained, along with pectin fractions of higher molecular weight in which virtually all of the neutral sugars were included (de Vries et al., 1982; Jarvis, 1984; Thibault, 1983). From this, it was concluded that there is an intramolecular distribution in which the neutral sugars are concentrated in blocks of more highly branched rhamnogalacturonan regions, separated by unbranched regions containing almost exclusively D-galacturonan residues (De Vries et al., 1986). For apple pectin fractions, de Vries et al. (1983) established that the galacturonan residues in the highly branched regions are almost completely methylated, while the unbranched regions are 70% methylated. Pectic enzymes, referring to the general class of enzymes which degrade pectic polysaccharides, are produced by higher plants and microorganisms. Pectinesterase (pectin pectylhydrolase, EC 3.1.1.11; older names: pectin methylesterase, pectin demethoxylase, pectin methoxylase, pectase) is the name given to the enzyme which catalyzes the hydrolysis of the methyl ester groups present in pectin. Polygalacturonases and the lyases (pectate lyase and pectin lyase) split the main galacturonan chain of pectic polysaccharides by hydrolysis and transelimination, respectively. Many of the chain-splitting enzymes require free carboxyl groups for activity and can only degrade polymers with a high content of methyl ester when they operate in conjunction with PE (Pilnik et al., 1973).

Occurrence of Pectinesterase

Pectinesterase was first described by Fremy in 1840 as being responsible for the coagulation of soluble pectin into a gel in the presence of calcium ions in vegetable juice. Since then, numerous higher plants and plant pathogenic microorganisms have been shown to be able to produce PE. Plants seem to contain PE in every tissue (Bell et al., 1951; Rexova-Benkova and Markovic, 1976), however, activity levels vary considerably with plant species, variety, component part of the plant, and stage of growth. The PE activity in fruits and vegetables during growth and ripening does not show any distinct trends. Activity levels have been reported to increase during ripening in orange (Rouse and Atkins, 1953a), tomato (Pozsar-Hajnal and Polacsek-Racz, 1975; Rathens and Krivilieva, 1987; Tucker et al., 1982), and banana (Hultin and Levine, 1965). Activity levels have also been shown to remain constant in banana (Brady, 1976), mango (Ashraf et al., 1981), and tomato (Sawamura et al., 1978). A decrease in activity was reported during ripening in tomato (Pressey and Avants, 1972), avocado (Rouse and Barmore, 1974), cucumber (Bell et al., 1951), mango (Roe and Bruemmer, 1981) and pear (Weurman, 1954). In many cases, these enzymes are present throughout fruit development and often show no correlation with softening. Huber (1983) suggested that the conflicting reports regarding the activity of PE during ripening is due to the sensitivity of these enzymes to polyphenolic compounds and cations. When precautions were taken to prevent enzyme inactivation by polyphenols, PE activity in ripening banana remained constant (Brady, 1976). Differential extractability during ripening may also contribute to apparent activity changes (Brady, 1976).

Pectinesterase was shown to be situated in the intercellular free space in oat coleoptiles and tobacco pith (Bryan and Newcomb, 1954; Glasziou, 1959). It is thought that PE is bound primarily by ionic interaction to cell-wall pectic substances since it can be removed by raising the pH above 7.0 and increasing the ionic strength, as was demonstrated previously in oranges (MacDonnell et al., 1945), oat coleoptiles (Glasziou, 1959) and tomatoes (Pozsar-Hajnal and Polacsek-Racz, 1975). Moreover, it has been demonstrated that there are specific binding sites for PE in tomato cell walls and oat coleoptiles (Jansen et al., 1960; Nakagawa et al., 1971). The situation appears to be complicated, however, as the existence of multiple forms of PE with different binding affinity for cell walls or pectic substances have been demonstrated (Glasziou and Inglis, 1958; Hultin and Levine, 1963; Versteeg et al., 1978; Wicker et al., 1988).

Role in Vivo

Since PE is present in all living tissues of higher plants, an essential function seems probable. Auxins such as 3-indolylacetic acid (IAA), which promote cell wall extension, have been shown to both stimulate (Glasziou and Inglis, 1958; Nakagawa et al., 1970) or inhibit PE activity (Bryan and Newcomb, 1954), depending on concentration. Glasziou and Inglis (1958) found that IAA promotes the binding of PE to cell walls of tobacco pith and artichoke tubers. Jansen and Jang (1960) reported that IAA had no effect on the degree of esterification of pectic material in the cell wall, thus arguing against the hypothesis that IAA promotes elongation of cells through a PE mediated effect. It is widely held that the initial wall loosening effect of auxin on plant stems is mediated by a fall in the pH of the cell wall (Cleland, 1981; Terry and Jones, 1981). Evidence has been obtained that the activity of PE generates fixed negative charges in the cell-wall of soybean, thus creating a Donnan potential at the cell surface and a local downward shift in pH (Moustacas et al., 1986). It has been proposed that the downward shift in pH indirectly produced by PE serves to activate cell-wall loosening enzymes (glycosyltransferases) involved in autolysis and cell-wall extension (Nari et al., 1986).

It has been thought for some time that PE is not of vital importance in fruit softening (McCready, 1955; Hobson, 1964). Significant PE activity has been found prior to softening

(Brady, 1976; Tucker et al., 1982) and in underdeveloped fruit (Pressey and Avants, 1982a). Furthermore, normal amounts of PE activity were reported in slow ripening (Nr) tomato mutants (Tucker et al., 1982). Since various forms of PE are present throughout tomato ripening (Pressey and Avants, 1972; Tucker et al., 1982), indiscriminate de-esterification might be expected to occur in vivo. This does not appear to be the case since in many instances the degree of methylation in total cell-wall pectic substances was reported to be quite high and remained constant during softening (Gee et al., 1959; Knee, 1978, Shewfelt, 1971; Wade, 1964). From these findings, it seems reasonable to conclude that either PE activity does not contribute to fruit softening, or that additional factors are needed.

Polygalacturonase (PG), which increases during ripening (Huber, 1983), has often been implicated in the cell-wall metabolism responsible for fruit softening, and furthermore, is not synthesized by tomato ripening mutants (Nr, rin). It has often been suggested that the function of PE in tissue softening during ripening or plant pathogenesis is to effect sufficient de-esterification of wall pectins to allow polygalacturonase to function. It is clear that de-esterification of highly methoxylated pectin enhances depolymerization by both endo- and exopectolytic enzymes (Dahodwala, 1974; Rexova-Benkova and Markovic, 1976). Pressey and Avants (1982b) found that very low levels of PE increased the solubilization by PG of isolated cell walls from tomato, while at high PE levels, solubilization was inhibited. Conversely, Versteeg (1979) found that PG greatly stimulated PE activity in vitro, presumably by removal of the inhibiting reaction product. In light of these facts, it seems reasonable to conclude that the activities of PE and PG are synergistic, and at least in tomato, both activities are needed for normal ripening.

If de-esterification of cell-wall pectic substances by PE does occur during ripening, effects beyond that of satisfying the substrate specificity of PG seem likely. Demethoxylation would create a situation in which the galacturonan chains, now bearing additional negatively charged groups, would be forced apart by electrostatic repulsion. Swelling of the galacturonan chains could conceivably facilitate the mobility of cell-wall loosening enzymes. The occurrence of this mechanism would depend on the availability of divalent cations, primarily calcium, which would act to negate the swelling. There have been reports that for a number of fruit types levels of bound calcium decreased drastically during ripening (Huber, 1983; Suwwan and Poovaiah, 1978; Tingwa and Young, 1974; Wills and Tirmazi, 1982). The suppression of ripening and respiratory climacteric by application of calcium to ripening fruit has also been reported (Huber, 1983; Tingwa and Young, 1974, Tirawzi and Wills, 1982). The role of calcium in regulation of cell wall hydrolase activity is still unclear, however.

Pectinesterase also appears to play a role in tissue softening during plant pathogenesis. Mutants of Erwinia chrysanthemi which lacked PE activity were noninvasive, demonstrating the important role of this enzyme in soft rot disease (Boccara and Chatain, 1989). Pectinesterase activity has also been shown to contribute to pathogenicity in strains of Fusarium oxysporum (Pequin and Coulombe, 1962) and Xanthomonas malvacearum (Abo-El-Dahab, 1964). Dreyer and Campbell (1984) showed that increased methylation of pectin hindered aphids in penetrating host-plant tissue, while aphids with higher PE activity levels are able to overcome this barrier.

There are indications that PE action alone (in the absence of PG) provides resistance against pathogens. Pectinesterase action and subsequent binding of pectate with divalent cations increases tissue firmness (Brown, 1969). In several cases, it has been demonstrated that host PE activity increases upon infection (Bateman, 1963; Langcake, et al., 1973). In bean hypocotyls infected by Rhizoctonia solani, calcium ions accumulate in the infected zone, host PE activity increases, and insoluble calcium-pectate is formed, which cannot be degraded by endo-PG of the pathogen (Bateman and Millar, 1966).

In several plants, multiple forms of PE have been demonstrated: four to eight in tomato (Delincee, 1976; Pressey and Avants, 1972), two in apple (Castaldo et al., 1989) two to six in banana (Brady, 1976; Hultin and Levine, 1963; Markovic et al., 1975), two in tobacco pith (Glasziou and Inglis, 1959), two in Chinese cabbage (Ko and Park, 1984), three in artichoke (Glasziou and Inglis, 1959), two to five in orange (Evans and McHale, 1979; Kornar et al., 1990; Versteeg, 1979), and up to twelve in other citrus varieties (Nieuwenhuis, 1974; Versteeg, 1979). That the two major isoenzymes of PE in Navel orange were found exclusively in juice sacs or peel suggests functional differences (Versteeg, 1979). Evans and McHale (1979) and Rombouts et al. (1982) also demonstrated differences in amounts of various PE forms in different tissues of citrus fruits. Tucker et al. (1982) reported changes in the ratios of the various tomato PE forms during ripening. The situation may be very complicated if the eight forms found in tomato or the twelve forms found in citrus have different functions in vivo. The multiple forms of PE always differ in isoelectric point and usually differ in one or more of the following: pH activity profile, activation by cations, substrate affinity, inactivation by detergents, inhibition by sucrose, heat stability, relative molecular mass, and binding affinity to pectic substances (Brady, 1976; Glasziou and Inglis, 1958; Hultin et al., 1966;

Hultin and Levine, 1963; Pressey and Avante, 1972; Versteeg et al., 1978)

Purification and Properties

While the presence of PE has been established in many plants and microorganisms, the properties of these enzymes have not been well described. They were purified partially or completely only in relatively few cases. Available information on plant PEs has been summarized by Versteeg (1979). Direct comparison of properties in most cases is of limited value, since assay conditions differed. The data on impure preparations usually represent the overall properties of a mixture of different forms of PE.

Purification of plant PE usually includes extraction, fractional ammonium sulphate precipitation or acetone precipitation and several types of column chromatography, such as gel filtration, or anion or cation exchange. Sometimes other principles were employed as well, for instance, affinity chromatography (Versteeg et al., 1978; Brady, 1976; Rexova-Benkova et al., 1977) and calcium phosphate absorption chromatography (Dahodwala et al., 1974; Yoshihara et al., 1977). A detailed summary of purification methods used for PE is given by Rexova-Benkova and Markovic (1976).

Plant PEs described to date possess molecular weights in the range of 24-37 kD, with the exception of one low molecular weight form from banana (Markovic et al., 1975) and high molecular weight forms in citrus (Versteeg, 1979) and apple (Castaldo et al., 1989). Plant PEs have pH optima ranging between 6.0 and 8.5 (Bell et al., 1951; Brady, 1976; Chang et al., 1965, Ko and Park, 1984; Lee and Macmillan, 1968; Lineweaver and Ballou, 1945; Miyairi et al., 1975; Moustacas et al., 1986; Puri et al., 1982; Versteeg, 1979, 1980). The isoelectric points of PEs are all above 7.0 (Brady, 1976; Delincee, 1976; Evans and McHale, 1978). Citrus PEs are especially basic proteins, having isoelectric points greater than 10 (Rombouts et al., 1982; Versteeg, 1978, 1979). Michaelis-Menten constants reported for pure enzymes varies over a range of greater than 500, however, the wide range probably in part reflects substrate variability, since no common substrate was used in these studies.

It has been demonstrated that one of the multiple forms of tomato PE was neither a glycoprotein nor lipoprotein (Markovic, 1974). Theron et al. (1977) found indications for a glycoprotein character of plum and tomato PE. None of the six banana PEs studied by Markovic et al. (1975) were lipoproteins. Versteeg (1979) demonstrated that the two major forms of PE in orange were not glycoproteins.

The optimal temperature of plant PE is usually between 50° and 60°C (Collins, 1970; Dahodwala et al., 1974; Ko and Park, 1984; Lee and Wiley, 1970; Miyairi et al., 1975; Montedoro, 1968, Puri et al., 1982). A 1.3 to 1.4 fold increase in reaction rate as temperature is increased by 10°C in the range of 20-50°C has been reported in most cases (Bell et al., 1951; Collins, 1970; Hills and Mattern, 1947; 1968; McColloch and Kertesz, 1947; Puri et al., 1982, Van Buren et al., 1962, Vas et al., 1967). Plant PEs are relatively heat stable enzymes, with inactivation temperatures falling in the range of 60-100°C (Arakji and Jang, 1969; Bell et al., 1951; Lee and Wiley, 1970; Manabe, 1973a; Markovic, 1978; Miyairi et al, 1975; Nakagawa et al, 1970; Versteeg et al., 1980).

Two forms of Navel orange PE were demonstrated to be isoenzymes: they had the same molecular weight (37 kD), but different isoelectric points and amino acid compositions (Versteeg et al., 1978). The isoenzymes, along with a high molecular weight form (54 kD), were detected in all citrus fruits, representing 85-95% of total activity (Versteeg, 1979). The inactivation temperatures of the isoenzymes were 60 and 70°C, while the high molecular weight form was inactivated at 90°C (Versteeg et al., 1980). These forms differed in the following properties: pH activity profile, K_m for pectin, K_i for polygalacturonic acid, stability in orange juice, heat stability, orange juice clarification properties, and temperature-activity relationship (Versteeg, 1979).

Specificity, Kinetics and Mode of Action

Plant PE is highly specific for methyl esters of (1-4)-α-D-galacturonan chains with a degree of polymerization of 10 or more (McCready and Seegmiller, 1954). Other esters (ethyl, propyl, allyl, and propargyl) of α-D-galacturonan are attacked at very low rates, and glycyl and glycerol esters are not de-esterified at all (Deuel, 1947, MacDonnell 1950; Manabe, 1973b) The methyl ester of alginate is not de-esterified by orange PE (MacDonnell et al., 1950). Although crude orange PE shows activity on alkyl esters of several acids, MacDonnell (1950) concluded that this is probably due to contaminating carboxylesterases other than PE Lower reaction rates are observed if part of the galacturonate ester units in the main chain have been reduced to galactose, if part of the secondary hydroxyls (at C2 and C3) are acetylated (Solms and Deuel, 1955), or if side chains of neutral sugars are present (Versteeg, 1979). Thus, an unobstructed D-galacturonan chain and the methyl ester appear to be essential for full activity. Therefore, it seems necessary to express the K_m of a PE on the basis of pectin concentration together with a good description of the pectin molecule (including anhydrogalacturonic acid content, degree of esterification, distribution of carboxyl groups and degree of polymerization).

De-esterification by plant PE proceeds linearly along the pectin chain so that blocks of free carboxyl groups are produced. This conclusion was reached by comparing randomly de-esterified pectins (from partial acid or alkali de-esterification) with partially enzymatically de-esterified pectin in regard to viscosity of solutions, calcium gel strengths, and electrophoretic and chromatographic patterns (Kohn et al., 1968; Lee and Macmillan, 1970, Schultz et al., 1945). A random mode of de-esterification has been demonstrated for Aspergillus PEs (Ishii et al., 1978 and 1979, Kohn et al., 1983; Kohn et al., 1985).

The activity of plant PE is maximal at pH 8.0 and decreases to a minimum around pH 3.5 (Moustacas et al., 1986, Versteeg, 1979). To explain this behavior, Moustacas et al., (1986) proposed a classical Dixon model, where two interconvertible enzyme ionization states coexist. However, since a free carboxyl group(s) is needed for binding (Solms and Deuel, 1955), it seems possible that the ionization state of the substrate may at least partially influence the pH-activity profile. This would explain the observed increase in K_m of Navel orange PE as the pH is lowered from 7.0 to 2.5 Versteeg, 1979). The observed decrease in V_{max} of Navel orange PEs over the same pH range (Versteeg, 1979) indicates protonation of acidic groups on the enzyme is occurring. Remarkably, the V_{max} of the high molecular weight PE of Navel orange increases from pH 4.0 to pH 7.0, and also increases from pH 4.0 to pH 2.5 (Versteeg, 1979). This characteristic was not apparent for the two low molecular weight isozymes of Navel orange (Versteeg, 1978). The apparent K_i for polygalacturonate, a competitive inhibitor of Navel orange PE, is reduced at lower pH values (Versteeg, 1979).

Different forms of PE from the same source may have different modes of action. Differences in orange juice-clarifying properties among Navel orange PE forms was demonstrated by Versteeg (1979). One form does not clarify juice even though methanol is produced at similar levels to other forms which destabilize cloud. Since pectate chains must have blocks of 16 or more monomers with free carboxyl groups for juice clarification (Termote et al., 1977), it seems possible that this enzyme produces shorter blocks of carboxyl groups than the other PE forms. Krop (1974) also observed differences in the clarification of fresh juices from different orange varieties which could not be related to overall PE activity.

On the basis of enzymatic activity on partially de-esterified pectin, Solms and Deuel (1955) concluded that orange PE hydrolyzes ester bonds adjacent to carboxyl groups and that carboxyl groups are needed for enzyme-substrate complex formation. On alkali de-esterified pectins, for both Navel orange PEs tested, the K_m values were reduced, but V_{max} values were largely unaffected by a decreasing the degree of esterification (DE) (Versteeg, 1979). The increase in affinity ($1/K_m$) is much greater than would be expected if only one carboxyl group is involved in enzyme-substrate complex formation; doubling the DE gives a 4-fold increase in $1/K_m$. From this data, it was deduced that two free dissociated carboxyl groups are needed at some fixed distance for enzyme-substrate complex formation (Versteeg, 1979). The decreased affinity at lower pH is in agreement with this theory. However, another type of enzyme-substrate complex seems possible because of the frequency of attack on highly esterified pectin chains as observed by Versteeg (1979).

Stimulation, Inhibition and Inactivation

The heat stability of PEs is discussed under the heading "Stability of Citrus Pectinesterase". Plant PE are competitively inhibited by pectate (Termote et al., 1977), Lineweaver and Ballou (1945) reported that cations but not anions stimulate PE on the acid side of the pH optimum. Inhibition by pectate was also lower at higher pH values and at higher cation concentrations (Lineweaver and Ballou, 1945). It was concluded that cations do not stimulate enzyme activity, but serve to liberate the enzyme from an inactive ionic complex with the product. Divalent cations stimulate PEs at much lower concentrations than monovalent cations (Lineweaver and Ballou, 1945; MacDonnell et al., 1945; Miyairi et al., 1975; Van Buren et al , 1962).

Octamers of pectate have been shown to competitively inhibit orange PE (Termote et al., 1977). Activity is stimulated by endo-pectate lyase and endo-polygalacturonase, presumably by removal of blocks of free carboxyl groups which act as competitive inhibitors (Versteeg, 1979). Oligomers of pectate have been successfully used to increase the cloud stability of the orange juice (Baker and Bruemmer, 1972; Termote et al., 1977). Tannic acid, an effective phenolic inhibitor of PE activity, has also been used to stabilize the cloud in orange juice (Ball, 1966). Several other polyphenols have also been shown to be inhibitors of PE activity (Mitek and Drzazga, 1988). Noncompetitive inhibition of plant PE by molar solutions of sugars and alcohols was reported (Chang et al., 1965) and Versteeg (1979) hypothesized that this effect was due to reduction of water activity. Reversible inhibition of PE activity in tomato by ethylene has been reported (Medina et al., 1981). Various anesthetics and dichlorodifluoromethane were shown to inhibit PE activity (Laverty and Fennema, 1985)

Irreversible and noncompetitive inhibition by iodine has been shown to increase with the purity of tomato and carrot PE preparations (Markovic, 1978; Markovic and Patocka, 1977), suggesting the presence of tyrosine at the active center. Tomato PE is also inactivated by diethyl-pyrocarbonate, suggesting the presence of histidine at the active center (Markovic and Jornvall, 1986). The effects of a wide variety of chemical agents on PE has been summarized by Versteeg (1979).

Stability of Citrus Pectinesterase

All citrus PEs have inactivation temperatures falling in the range of 60-90°C. In previous literature, irregular heat inactivation patterns of crude citrus PE have been shown (Bissett et al., 1953; Rouse and Atkins, 1952, 1953b). Discrepancies between heat stability properties of two PEs purified from Navel orange and crude preparations from the same source have also been reported (Versteeg, 1979). The crude PE contained a small fraction (5%) of activity which required a 20-30°C higher temperature for inactivation than the other purified PEs. Subsequently, a "high molecular weight" (HMW) PE was isolated and shown to be responsible for this heat stable activity. The three molecular forms in Navel oranges have considerably different inactivation temperatures; 60, 70, and 90°C after 5 min of heating. Differences in heat stability of PE in citrus juices at the same pH, °Brix, ion concentrations and pulp content (Eagerman and Rouse, 1976; Rouse and Atkins, 1952, 1953) now can be ascribed to the presence of different forms of PE.

Storage stability of the PEs from Navel orange in a simulated orange juice environment (pH 4) at 6°C and 30 °C, and the effects of juice constituents on PE stability was examined by Versteeg (1979) The HMW PE (See purif. and prop.) appeared to be indefinitely stable in these conditions, unlike the two low MW PE forms, each of which was reduced tenfold in either 0.22 or 21 days. Varying juice constituents had no effect on HMW PE; however, an increase in sugar concentration increased the stability of the low MW forms, while an increase in ionic strength had a destabilizing effect. Pectin concentration had no effect on stability of any of the forms.

Bissett et al. (1953) observed a complicated relationship between heat stability of PE, concentration factor, and cloud stability of Valencia juice concentrates: PE was less heat stable in 2- to 4- fold concentrated juice than in single strength juice, but more heat stable in 6-fold concentrated juice. Cloud stability was greatest in 6-fold concentrated juice, however. Inhibition of low MW PE by the high sugar concentration could explain the increased cloud stability at the higher concentration factor.

Importance of Pectinesterase in Food Technology

Both desirable and undesirable effects before, during or after processing may be produced by endogenous PE activity in fruits and vegetables. In fresh or underpasteurized citrus juices, low methoxyl pectin produced by PE activity is precipitated by calcium ions, resulting in cloud loss of single strength juice or gelation of frozen concentrates. Both of these are serious quality defects because of negative effects on appearance, taste, aroma, and increased sensitivity to oxidation (Joslyn and Pilnik, 1961; Rouse and Atkins, 1952). Gelation of papaya puree has also been attributed to PE activity (Chang et al., 1965)

Of the three forms of Navel orange PE, the HMW PE has been shown to be the only form which effects significant clarification of chilled citrus juice and therefore the only form which can play a role in the loss of cloud of under-pasteurized citrus juices. The other forms of PE are rapidly inactivated at temperatures less than 80°C, the pasteurization temperature required to prevent microbial spoilage (Bissett et al., 1953), and to inactivate peroxidase (Nath and Ranganna, 1977), an enzyme which is responsible for flavor changes (Bruemmer, 1976). The severe time/temperature requirement to inactivate Navel orange PE, 0.8 min at 90°C (Versteeg, 1980), can cause cooked off-flavor (Kew and Veldhuis, 1961).

Endogenous PE activity can be used for protecting and improving the texture and firmness of several processed fruits and vegetables (Hsu et al., 1965; Lee et al., 1979; McFeeters et al., 1985; Voragen and Pilnik, 1989; Wiley and Lee, 1970). Blanching times and holding temperatures, sometimes with the aid of calcium ions or pH shift, are chosen so as to activate PE, causing partial de-esterification of pectins which then form ionic bridges, resulting in stronger intracellular adhesion. The heat applied for blanching serves to free PE from the cell wall (Pilnik, 1981).

Pectinesterase is usually an integral part of the commercially available pectolytic fungal enzymes used to clarify, reduce viscosity, and increase press yields of juices (Baron and Drilleau, 1982; Gonzalez et al., 1977; Nelson and

Tressler, 1980; Rombouts and Pilnik, 1978), and liquefy fruits (Voragen et al., 1985)

Citrus and tomato PE have also been used to produce low methoxyl pectin which is used to make gels with low or no sugar content (Lee and Taylor, 1962), commonly used in dietetic jellies. However, low methoxyl pectin produced by plant PEs instead of by acid de-esterification gives a weaker calcium pectinate gel (Hills et al., 1949).

A limitation to the use of PE in fruit juice and wine technology is the production of methanol. Methanol concentrations can rise to 400 mg/L and concentrations of 50 mg/L are quite common (Baumann and Gierschner, 1974). The methanol in distilled spirits from fruits is also caused by PE activity (Tanner, 1970). Methanol in fresh fruit (also due to PE activity) has been reported at 10-20 mg/L but can rise to nearly 200 mg/L (Segal et al., 1966).

Structure-Stability Relationship in Proteins

It has been well established that enzymes from thermophilic microorganisms generally have higher intrinsic stabilities than those of mesophilic organisms. The primary, secondary, tertiary, and where appropriate quaternary structural parameters of thermophilic enzymes are in general strikingly similar to mesophilic proteins having the same function. In spite of their similar structural parameters, thermophilic enzymes are invariably more stable to denaturing conditions than their mesophilic counterparts. Amino acid sequence analysis of thermophilic and mesophilic enzymes suggests that enhanced thermostability is due to a small number of amino acid replacements (Stellwagen and Wilgus, 1978). In general, the types of amino acid substitutions proposed to enhance stability of enzymes include the increased presence of amino acid residues capable of forming additional intramolecular bonds (disulfide, hydrophobic, hydrogen, or ionic), complementary intersubunit contacts, tighter binding to prosthetic groups, or producing conformations having larger and more densely packed protein interiors (Mozhaev and Martinek, 1984).

The most important mechanism of thermostability enhancement is strengthening of hydrophobic interactions in the interior of the protein globule. Merkler et al (1981), in analyzing over 20 functionally different proteins from closely related thermophilic and mesophilic organisms, found a correlation between protein stability, and both the hydrophobicity scale of Nozaki and Tanford (1970), and the ratio of the volumes of polar to nonpolar amino acids (Fisher, 1964). Hydrophobic interactions play an especially important role in thermostability enhancement due to the fact that these forces increase with temperature, at least up to 60-70°C, unlike other noncovalent intramolecular forces (Scheraga, 1961). A simple correlation between hydrophobicity and stability is not always the case. Another scale of hydrophobicity, was suggested by Prabhakaran and Ponnuswamy (1980), which takes into account the frequency that a particular residue will be present inside the protein globule. From an analysis of data on 21 proteins with known primary and tertiary structures, the authors found that bulky, sterically hindered aromatic residues (although more hydrophobic on the Tanford scale) are less likely to be buried inside the protein than smaller, more flexible, aliphatic residues. Therefore, on this scale, valine, since it is more likely to be buried inside the protein, is more hydrophobic than tryptophan. This would explain the observed correlation between the content of aliphatic amino acids in thermophilic proteins (compared to mesophilic proteins) and protein stability (Biffen and Williams, 1976; Mozhaev and Martinek, 1984; Muramatsu and Nosoh, 1971; Singleton et al., 1969; Sundaram et al., 1980; Wedler et al., 1976). In some thermophilic proteins, the rise in content of aliphatic amino acids parallels a decrease in content of aromatic amino acids, so that the overall hydrophobicity on the Tanford scale may be the same or even less than their mesophilic analogs (Biffen, 1976; Wedler et al., 1976).

The compactness of the protein structure has also been suggested to enhance stability since destabilizing internal water molecules are pressed out of the interior of the protein globule. Substitution of an amino acid for a bulkier one in thermophilic proteins relative to corresponding mesophilic proteins had no effect on conformation or total volume of the enzymes, hence the thermophilic proteins are more compact (Argos et al., 1979).

Other alterations of amino acid composition which have been shown to influence thermostability include decreased content of cysteine, asparagine, and aspartic acid as well as the substitution of arginine for lysine and nonpolar residues for serine or threonine. The relationship between enzyme stability and cysteine content is apparently due to inactivation by the oxidation of sulfhydryl groups (Katchalski-Katzir and Freeman, 1982). The less accessible cysteine is to the solvent, the more stable the enzyme will be to an oxidative-type inactivation. Since additional disulfide bonds have been shown to increase enzyme stability in some cases (Wedler et al., 1976), it appears that stability is affected by the content of free sulfhydryls and not total cysteines. Both arginine and lysine are located primarily on the protein surface (Shulz and Schirmer, 1979) and hence have contact with water. These residues contain relatively large hydrocarbon fragments whose contact with water is thermodynamically unfavorable. If lysine is replaced by arginine, the area of unfavorable contact decreases, first because the hydrocarbon side chain is one CH_2 group shorter, and second because of better shielding of the hydrophobic part of the residue by the large guanidine group of arginine (Mozhaev and Martinek, 1984). It has been reported that peptide bond hydrolysis adjacent to aspartic acid occurred at rates comparable to those of irreversible thermoinactivation (Zale and Klibanov, 1984). This would explain the observed enhancement of protein stability by replacement of aspartic acid with glutamic acid (Argos et al., 1979). Another process leading to thermoinactivation of enzymes involves the deamidation of asparagine and possibly glutamine residues. Enzymes having electrostatic affinity for negatively charged substrates will be adversely affected by deamidation, whereas enzymes whose activity is unaffected by increasing the negative surface charge or whose composition is low in asparagine will be relatively more stable at all pH values (Ahern and Klibanov, 1985; Zale and Klibanov, 1984). Lack of polar amino acids, mostly serine and threonine, in thermophilic proteins has been reported (Mozhaev and Martinek, 1984). These two residues are generally located not only on the protein surface but also in the interior. It has been calculated that the localization of these polar residues inside the protein is thermodynamically unfavorable if no additional intramolecular hydrogen bonds are formed by the polar group (Shulz and Schirmer, 1979). In this case, the substitution of the interior serines and threonines with nonpolar residues should increase the stability of proteins.

Likewise, substitution of surface serines or threonines with nonpolar residues should decrease stability.

The stability of some enzymes has been shown to increase considerably in the presence of substrates or prosthetic groups (Wedler et al., 1976). Stronger binding of prosthetic groups has been correlated with enhanced protein stability as well (Devanathan et al., 1969; Hon-nami et al., 1979).

Glycosylation of enzymes has also been shown to enhance stability (Chu et al., 1978; Hayashida and Yoshioka, 1980; Tashiro and Trevithick, 1977). The mechanism of stabilization by carbohydrate has not been determined; however, Chu et al. (1978) obtained evidence which indicates that the carbohydrate moiety of yeast invertase promotes the correct refolding of the denatured enzyme in vitro. This leads to the conclusion that carbohydrate chains may direct the folding of proteins to their most stable three dimensional conformation in vivo

MATERIALS AND METHODS

Preparation of Crude Extracts

Thermolabile Fraction

For purification of thermolabile pectinesterase (TL PE), crude extract was prepared by the method of Wicker et al. (1987). Marsh white grapefruit pulp (Citrus World, Lake Wales, FL or Golden Gem, Umatilla, FL), 300 gm per purification run, was homogenized in a 1:5 ratio (w/v) of pulp to 0.25 M Tris-chloride buffer, pH 8, with 0.3 M sodium chloride, 1 mM sodium azide, for 1 min in a blender. The mixture was stirred for 1 h at 4°C, then centrifuged at 16,000 X g for 25 min at 4°C. To the supernatant, solid ammonium sulfate to 75% saturation was added at 4°C. After standing overnight at 4°C, the precipitate was collected by centrifugation at 10,000 X g for 20 min at 4°C. The solution was dialyzed against 4 changes of 10 volumes of 10 mM sodium phosphate buffer, 1 mM azide, pH 7.5.

Thermostable fraction

Crude extract for the purification of thermostable pectinesterase (TS PE) was prepared by a modification of the method of MacDonnell et al. (1945) and Krop (1974). To Marsh white pulp, 3 kg per purification run, was added two parts buffer containing 0.25 M tris-chloride, 0.3 M sodium chloride, and 1 mM sodium azide, at pH 8.0. The mixture was homogenized for 1 min in a blender, then stirred 1 hour at 4°C. The homogenate was centrifuged at 16,000 x g for 20 min and solid ammonium sulfate was added to 30% saturation. After standing overnight at 4°C, the solution was centrifuged at 10,000 x g for 20 min at 4°C. The pellet and the floating oil layer were discarded. Solid ammonium sulfate was added to the supernatant to 75% saturation. After standing overnight, the precipitate was collected by centrifugation at 10,000 x g for 20 min at 4°C. The precipitate was redissolved in 10 mM sodium phosphate buffer, pH 7.0, with the aid of an ultrasonic bath, and then dialyzed against 3 changes of 20 volumes of the phosphate buffer at 4°C. After dialysis, 1 mM sodium azide was added to the enzyme solution as a preservative.

The crude PE solution was then heat treated at 70°C for 5 min to obtain the thermostable PE fraction. The enzyme solution was heated in aliquots of 30 mL in large (25 mm) test tubes. Solutions were rapidly brought up to 70°C by placing in a waterbath adjusted to 85°C and then shifted to a 72°C waterbath for 5 min. The supernatant was collected by centrifugation at 10,000 x g for 20 min.

Enzyme Assays

Titrimetric Assay

Pectin solutions for titrimetric assays were prepared as follows: 0.1 M sodium chloride and 1 mM sodium azide solution was heated to 40°C. Pectin (for a final concentration of 1%) was slowly added slowly while blending. In standard assays, PE solution is added to 25 mL of the 1% pectin solution at 25°C, pH 7.0 or 7.8, depending on the pH optimum of the enzyme being assayed. While stirring with a magnetic stirrer, the initial reaction velocity is measured by automatic titration of the liberated carboxyl groups with standardized 0.05 N sodium hydroxide in a Combi recording pH-stat (Metrohm Ltd., Herisau, Switzerland). One unit of PE activity was defined as the amount of enzyme which liberates 1 µmole of carboxyl groups per min at standard assay conditions.

Spectrophotometric assay

The spectrophotometric assay for plant pectinesterases of Hagerman and Austin (1986) was determined to be useful for the measurement of relative activities but not absolute activities due to high variability of the standard curves. Therefore a modification of this method was used to assay column chromatography fractions only. A 0.5% solution of Sunkist pectin containing 0.1 M sodium chloride, 1 mM sodium azide was prepared as described for the titrimetric assay. A 0.04% solution of bromthymol blue (Sigma Chemical Company, St Louis, MO) was prepared in distilled water. Just before assays were carried out, 7.5 mL of bromthymol blue was mixed with 100 mL of the pectin solution and the mixture adjusted to pH 7.8 with sodium hydroxide. Distilled water was then added to make a total volume of 150 mL (check pH again). To a cuvette was added 1.5 mL of the mixture; the initial A_{620} was 1.1-1.2 vs. a water blank. The reaction was started by addition of up to 20 µL of PE solution to the cuvette and the rate of decrease in A_{620} over time was recorded in a Beckman DU7 spectrophotometer (Beckman Instruments Inc., Palo Alto CA) The assay was calibrated with galacturonic acid and the decrease in A_{620} is linear when up to 0.2 µmol of acid is added per 1.5 mL of pectin solution. Solution pH must be closely monitored to ensure reproducible color changes. Buffers interfere with the measurement of acid production, so the reagents must be prepared as unbuffered or very weakly buffered solutions.

Protease assay

Crude extracts were assayed for protease activity by the method of Lawrence and Sanderson (1969). This method is based on radial diffusion of enzymes into a skim milk containing agarose gel. A 1% solution of agarose, 0.5 g, (Bio-Rad Laboratories, Richmond, CA) was prepared in 50 mL of boiling distilled deionized water. While still liquid, 7.2 mL of the agarose was mixed with 0.8 mL of a 3.3% solution of skim milk powder, 0.1 M sodium acetate buffer, pH 6.0, and the mixture was then poured into petri dishes. After solidification, the petri dishes containing the gel were immersed in a large beaker containing 300 mL of 0.1 M sodium acetate, at either pH 4.5 or 6.0 for 2 h. The petri dishes were removed and wells 5 mm in diameter were punched in the substrate gel and 10 µL aliquots of enzyme solution were added. The enzymes tested were: 1) crude extract (prepared by the method of Wicker et al., 1987), 2) crude extract after ammonium sulfate precipitation and cation-exchange chromatography, 3) standard protease solutions containing either 3000 u/mL of pepsin (Sigma Chemical Company) or trypsin (Sigma Chemical Company). A control of 0.1 M sodium acetate buffer, pH 4.5 or 6.0 was also applied to the wells. The gels were incubated at room temperature for 24-48 h, then examined for clearing zones.

Protein Assays

Protein concentrations were estimated by the method of Smith et al (1985). A working reagent is prepared in the following manner: an aqueous solution of 1% bicinchoninic acid disodium salt, 2% sodium carbonate, 0.16% disodium tartrate, 0.4% sodium hydroxide, and 0.95% sodium bicarbonate, pH 11.25 (Pierce Chemical Company, Rockford IL) was mixed at 50:1 with an aqueous solution of 4% cupric sulfate pentahydrate (Pierce Chemical Company, Rockford, IL). The reagent solutions are stable indefinitely at room temperature; the working reagent is stable for 1 week. The assay procedure consists of mixing 1 volume of sample (1-40 µg protein) with 20 volumes of working reagent in a test tube. Tubes were incubated at room temperature for 24 h, and then the absorbance was read at 562 nm in a Beckman DU 40 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) using BSA as a standard.

Relative concentrations of protein in chromatography eluent fractions was estimated from the A_{280}. Measurements were carried out in quartz cuvettes (1 cm) in a Beckman DU7 spectrophotometer. If necessary, protein solutions were adjusted so that the absorbance was within the 0.1-1.0 range.

Pectin Analysis

Pectins used in kinetic analysis were obtained from Sunkist Growers, Inc., (Ontario, CA; Lot # A7216), from Eastman Kodak Company (Rochester, NY; Lot # P2569) and from H.P. Bulmer Ltd (Hereford, England; Samples A,C,D,E,F).

Sunkist pectin was used in the determination of K_m and V_{max}; therefore this substrate was characterized completely with respect to degree of esterification (DE), anhydrogalacturonic acid (AGA) content, and degree of polymerization (DP). Kodak, Bulmer, and Sunkist pectins were used to investigate the effects of DE on activity, therefore, it was necessary to determine the DE and AGA content of Kodak pectin. The DE of Bulmer pectin was provided by the manufacturer.

Degree of Polymerization of Pectins

The DP of Sunkist pectin was estimated viscosimetrically. The viscosity of 4 pectin concentrations (2.5, 1.25, 0.625, 0.312 g/L) in 0.1 M Tris-succinate buffer, pH 6, with 0.2 M sodium chloride was measured in a Cannon-Fenske Reverse Flow Viscosimeter No.551, size 100, kept in a 40°C waterbath. The intrinsic viscosity was determined from the y-intercept of a plot of the ratio of specific viscosity to concentration against concentration and by extrapolating to zero concentration. The formula $[\eta] = 1.4 \times 10^{-6}\ MW^{1.34}$ was used to calculate the average molecular weight (Owens et al. 1946). To get the average DP, the MW of the pectin was divided by the average MW of the monomers (176-190, depending on the DE).

Anhydrogalacturonic Acid Content of Pectins

The AGA content of Sunkist and Kodak pectins were determined by the method of Blumenkrantz and Asboe-Hansen

(1973). To an aliquot of pectin (10-50 μg in 500 μL) was added 5 volumes (2.5 mL) of sulfuric acid containing 0.25% sodium tetraborate. The mixture was vortexed, kept on ice for 5 min, then boiled for 10 min. After cooling on ice, 50 μL of 0.15% m-phenylphenol in an aqueous solution of 0.5% sodium hydroxide was added to the first tube to be read. After vortexing lightly, the solution was poured into a cuvette (down the sides to prevent bubble formation), and absorbance at 520 nm was read on a Beckman DU 40 spectrophotometer when color reaches a maximum (about 2-5 min). Galacturonic acid (1-20 μg in 500 μL water) was used as a standard.

Degree of Esterification of Pectins

The DE of Sunkist and Kodak pectins were estimated by the method of Wood and Siddiqui (1976). Pectin (100-250 μg in 0.5 mL) was saponified by adding 0.25 mL of 1.5 N sodium hydroxide. After 0.5 h at room temperature, the samples were acidified by 0.25 mL of 5.5 N sulfuric acid and cooled in an ice-water bath. Aliquots of saponified pectin or methanol standard solution (2-40 μg) was adjusted to 1 mL volume, in 1 N sulfuric acid and cooled on ice. Then, 0.2 mL of 2% potassium permanganate was added and the solutions were mixed by gentle swirling and held in an ice-water bath for 15 min. Then, 0.2 mL of 0.5 M sodium arsenite in 0.12 N sulfuric acid was added, followed by the addition of distilled water, (0.6

mL), and the thoroughly mixed solution was left for 1 h at room temperature. Two mL of 0.02 M acetyl acetone dissolved in 2 M ammonium acetate and 0.05 M acetic acid was then added and, after shaking, the tubes were closed with marbles, heated at 60°C for 15 min, and cooled to room temperature. Absorbance at 412 nm was read, using a blank of water treated identically to the samples. To provide control solutions, potassium permanganate and sodium arsenite solutions were premixed and added to saponified, acidified pectin samples followed by acetyl acetone as usual. The DE was calculated from the concentration of methanol released by saponification after correcting for AGA content (Pilnik, 1981), as determined by the Blumenkrantz and Asboe-Hansen assay.

The following characteristics were found for Sunkist pectin: DE, 70%; AGA content, 69%; DP, 327. The following characteristics were found for Kodak pectin: DE, 62%; AGA content, 64%. The DP was not determined for Kodak pectin.

Column Chromatography

Thermolabile Pectinesterase

All purification steps were carried out at 4°C. The 75% ammonium sulfate thermolabile fraction, dialyzed against 10 mM sodium phosphate, pH 7.5, was adsorbed onto CM-Sephadex C50 (200 mL bed volume), preequilibrated in 10 mM sodium phosphate, pH 7.5, in a beaker. The adsorption was continued until less than 5% of the original activity was present in the supernatant (1-2 h), then the supernatant was decanted and the CM-Sephadex washed with 1000 mL of 10 mM sodium phosphate buffer, pH 7.5. After adsorption, the resin was packed in a 2.6 x 40 cm column and washed at a flow rate of 25 mL/h until A_{280} was less than 0.1. Enzyme activity was eluted with 0.2 M sodium chloride at pH 7.5 at a flow rate of 25 mL/h. Fractions of 6 mL were collected and assayed for PE activity (spectrophotometrically) and protein (by A_{280}). Fractions with the highest specific activity were pooled, and assayed for activity (titrimetrically) and protein (method of Smith et al.). The solution was then applied to a Sephacryl-200 gel filtration column (2.6 X 90 cm) and eluted at 3 mL/h with 10 mM sodium phosphate, 0.2 M sodium chloride, pH 7.0. Fractions of 1.5-2 mL were collected and assayed for PE activity and protein (by A_{280}). Fractions with the highest specific activity were pooled, and assayed for activity titrimetrically and for protein by the method of Smith et al. (1985). Pooled fractions were evaluated for purity by SDS-PAGE and by HPLC gel filtration as described in the characterization section.

Thermostable Pectinesterase

All purification procedures were carried out at 4°C. The 30-75% ammonium sulfate thermostable fraction, dialyzed against 10 mM sodium phosphate, was adsorbed onto CM-Sephadex C50 (200 mL bed volume), preequilibrated in 10 mM phosphate buffer, pH 7.0, in a beaker. The adsorption was continued until less than 5% of the original activity was present in the supernatant (1-2 h), then the supernatant was decanted and the CM-Sephadex washed with 1000 mL of sodium phosphate buffer, pH 7.0. After adsorption, the resin was packed into a 5 x 9 cm column and washed at a flow rate of 25 mL/h until A_{280} was less than 0.1. Enzyme was eluted by increasing the sodium chloride concentration linearly up to 300 mM over 1000 mL, at a flow rate of 25 mL/h. Fractions of 7-8 mL were collected and assayed for PE activity and protein (by A_{280}). Fractions of the highest specific activity were pooled for further purification, and assayed for activity (titrimetrically) and protein (method of Smith et al., 1985). After dialyzing to remove sodium chloride, the solution was adsorbed in a beaker onto CM-BioGel A, preequilibrated in 10 mM sodium phosphate buffer, pH 7.0, for 1-2 h at 4°C. The resin was packed in a 2.6 x 7 cm column and washed at a flow rate of 50 mL/h until the A_{280} of the eluant was less than 0.05. Enzyme was eluted by increasing the sodium chloride concentration linearly to 100 mM over 1000 mL, at a flow rate of 50 mL/h. Four-mL fractions were collected and assayed for PE activity and protein (by A_{280}). Fractions with the highest specific activity were pooled and assayed for activity (titrimetrically) and protein (method of Smith et al., 1985).

The solution was concentrated in an Amicon stirred-cell equipped with a PM 10 membrane (10 kD cutoff) to 11 mL and applied to a Sephadex G-75 column (2.6 x 95 cm) and eluted at 2 mL/h. Fractions of 1-1.5 mL were collected and assayed for PE activity and protein (by A_{280}). A pool of the fractions of highest specific activity were assayed for activity (titrimetrically), protein (method of Smith et al., 1985) and analyzed for purity by SDS-PAGE and HPLC gel filtration as described in the characterization section.

Molecular Characterization of Purified Pectinesterases

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out by the method of Laemmli (1970). Separations were performed using a Bio-Rad Protean II vertical slab unit for western blotting or a Bio-Rad mini-gel unit for analysis of purity and molecular weight. Running gels (10 mL for mini-gel and 20 mL for large slab) were composed of 12% acrylamide, 0.3% bis (both Bio-Rad electrophoresis grade), 0.1% SDS, and 0.03% ammonium persulfate in 0.375 M Tris-chloride, pH 8.8. Polymerization (overnight) was initiated by addition of 10 µL TEMED. A stacking gel, composed of 1% acrylamide, 0.03% bis, 0.1% SDS, and 0.1% ammonium persulfate in 0.125 M Tris-chloride, pH 6.8, was also used. Polymerization was carried out 1-2 h before sample application by addition of 5 μL TEMED. Proteins were diluted 4:1 with sample buffer (0.065 M tris-HCl buffer, pH 6.8, containing 10% glycerol, 1% SDS, 0.005% bromphenol blue) and heated at 100°C for 5 min. To check for the presence of sulfhydryl-linked subunits in one experiment, 1% 2-mercaptoethanol was added to the sample buffer. Proteins were loaded onto 0.78 mm thick large slab gels or 1 mm thick minigels and electrophoresed in running buffer of 0.6% Tris, 2.9% glycine and 0.1% SDS, pH 8.3. Current was kept constant at 10 mA per slab until the dye front entered the stacking gel, then the current was increased to 25 mA per gel. Following electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Eastman Kodak) and 0.1% Amido black (ICN Biomedicals, Costa Mesa, CA) in 40% ethanol, and 7% acetic acid, and then destained in 7% acetic acid. Electrophoretic patterns were recorded by developing the positive image using Kodak Electrophoresis Duplicating Paper (Eastman Kodak) or by photographing directly. Molecular weights of the protein bands were determined according to the method of Weber and Osborn (1969) and Weber et al. (1972) using a low MW standard kit (Pharmacia Fine Chemicals, Piscataway, NJ) containing: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (21 kD), and α-lactalbumin (14 kD).

For analysis of cyanogen bromide cleavage products, the standard Laemmli procedure was modified according to Giulian et al. (1985). Both the running gel (18%T, 0.5%C) and stacking gel (10% T, 4.8% C) contained 10% glycerol to slow diffusion and retard migration. The pH of the running gel was 9.3 instead of 8.8 as in the Laemmli system. Electrophoresis was carried out on a large slab gel under identical running conditions as described above, and stained for protein as described above. Cytochrome C (12.4 kD, Sigma, C 7150), ribonuclease A (13.7 kD, Pharmacia) and a-lactalbumin (14.4 kD, Sigma, L 4385), 10 μg each, were used as standards.

Dansyl Hydrazine Carbohydrate Staining on SDS Gels

To test for the possibility that the enzymes were glycosylated, staining for carbohydrate on SDS-PAGE gels was carried by the method of Eckhardt et al. (1976). Duplicate minigels loaded with 5 μg of PEs along with 5 μg each of a glycoprotein (ovalbumin, Sigma A2512) and non-glycoprotein (chymotrypsinogen, Sigma C 4879) as controls, were run as described in the SDS-PAGE methods section. Gels were fixed overnight in an aqueous solution (100 mL at all steps) containing 40% ethanol, 5% acetic acid (v/v). One gel was oxidized with periodic acid at room temperature by soaking gels in 0.7% paraperiodic acid in 5% aqueous acetic acid (v/v) for 2 h. this step was omitted for the duplicate gel in order to rule out erroneous results from the natural fluorescence of some proteins or binding of dansyl hydrazine to proteins by noncovalent forces. The gels were rinsed with distilled water and treated with 0.5% sodium metabisulfite in 5% aqueous acetic acid for 1.5 h until colorless in order to destroy excess periodic acid. Next, gels were placed in a freshly made solution of dansyl hydrazine (1mg/mL, Sigma) in acidified dimethyl sulfoxide (0.1 mL HCl/L DMSO) for 2 h at 60°C in a closed container. The dansyl hydrazine-dimethyl sulfoxide solution was poured off and gels were placed in a solution of 0.2 mg/mL sodium borohydride in dimethyl sulfoxide for 0.5 h at room temperature. Gels were rinsed with distilled water and destained overnight in 1% aqueous acetic acid with several changes; destaining was complete when the gel background was colorless under illumination with uv light. Glycoproteins were visualized with a long wavelength uv transilluminator (UV products, San Gabriel, CA) with a peak output at 366 nm.

Isoelectric focusing

Premade agarose isoelectric focusing gels (IsoGel), pH range 3-10, 0.6 mm thick, were purchased from FMC Bioproducts (Rockland, Maine). Purified PEs and isoelectric focusing standards (Broad pI kit, pH 3-10, Pharmacia) were loaded onto strips of blotting paper (Schleicher and Schuell Inc., Keene, NH) placed in the center of the gel and run at constant power for 2 hours at 8 W (500 V limiting) on a Hoefer Isobox flatbed system (Hoefer Inc., San Francisco, CA), using 10 mM phosphoric acid as the anode solution and 20 mM sodium hydroxide as the cathode solution. Gels were stained with 0.25% Coomassie blue/0.1% amido black and destained with 7% acetic acid.

HPLC gel filtration

Aliquots of 200 µL containing about 100 µg of the purified enzymes were subjected to HPLC gel filtration on a Superose-6 HR 10/30 column (Pharmacia Fine Chemicals) fitted to a Dionex system (Dionex, Houston, TX). Elution was conducted at room temperature with a buffer of 10 mM sodium phosphate, pH 7.0, containing 200 mM sodium chloride, at a flow rate of 0.3 mL/min and absorbance was read continuously at 254 nm in an 8 mm light path. Fractions of 100 µL were collected and assayed for pectinesterase activity as described previously. The native molecular weight of the PEs was determined based on regression analysis of the retention times of Pharmacia gel filtration standard proteins (Pharmacia Fine Chemicals, Piscataway, NJ). These were bovine serum albumin, 67 kD; egg albumin, 44 kD; carbonic anhydrase, 29 kD; and chymotrypsinogen, 25 kD. Standards were run individually under identical conditions as the PEs.

Ultraviolet Spectroscopy

The ultraviolet spectra of the purified enzymes was obtained by scanning from 220-400 nm in a Beckman DU7 UV-Vis spectrophotometer. The scans were carried out in quartz cuvettes of 10 mm path length in 10 mM sodium phosphate buffer, pH 7.0.

Analysis of Amino Acid Composition and N-Terminal Sequence

Purified PEs were first separated by SDS-PAGE using a Protean II vertical slab unit as described in the SDS-PAGE section. After electrophoresis, the separated proteins were electroblotted onto polyvinylidene difluoride membranes by the method of Matsudaira (1987). Gels were soaked in 200 mL of 10mM 3-[cyclohexylamino]-1-propanesulfonic acid (Eastman Kodak), 10% methanol, pH 10.7 for 5 min to reduce the amount of Tris and glycine. During this time a PVDF membrane (Millipore Corp., Bedford, MA) was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between the sheet of PVDF membrane and several sheets of blotting paper, was placed in a blotting apparatus (Bio-Rad) and electroeluted for 1.5 h at 50 V (0.75mm gel) in transfer buffer. The PVDF membrane was washed in deionized water for 5-10 min, stained in 0.1% Comassie Blue R-250 in 50% methanol for 5 min and then destained in 50% methanol, 10% acetic acid for 5-10 min at room temperature. The membrane was finally rinsed in deionized water for 5-10 min, air dried, and stored at -20°C. Protein bands corresponding to the R_f of the PEs were cut out with a clean razor and hydrolyzed in 6 N HCl under N_2 in sealed tubes for 24 h at 110°C. The content of cystine plus cysteine was obtained by conversion to cysteic acid by carrying out the hydrolysis in the presence of dimethyl sulfoxide. Tryptophan was determined after hydrolysis in 3 N mercaptoethane sulfonic acid. Typically, 0.3-0.4 nmol of proteins were recovered from 3-4 nmol of purified protein after the electroblotting and hydrolysis procedures for amino acid analysis. Amino acids were resolved and quantified using a Beckman 6300 amino acid analyzer with a Nelson analytical data acquisition system. Amino acid analyses were carried out by B. Parten at the Protein Chemistry Core facility, University of Florida.

In order to determine the N-terminal amino acid sequence of TL PE, about 0.3 nmol of TL PE, obtained from an electroblot of an SDS-PAGE gel as described above, was subjected to 10 cycles of Edman degradation on an applied Biosystems Model 470A gas phase protein sequencer. The PTH-amino acids were identified and quantitated using reverse-phase HPLC on a Novapak C-18 column. The analyses was also carried out by B. Parten in the Protein Chemistry Core facility. The protein remaining in the sample after Edman degradation was hydrolyzed and analyzed for amino acid composition as described above.

Cleavage of Pectinesterases with Cyanogen Bromide

Cyanogen bromide cleavage was carried out by the method of Gross and Witkop (1962). Formic acid (95%) was added to purified preparations of PEs (50 μg each) to obtain 66% formic acid, and the preparations were kept overnight at room temperature. A 100-fold excess of cyanogen bromide (100 mmol) (Sigma) was added to each preparation and the containers were capped and left at room temperature for 2 days. Cleavage products were solubilized in Laemmli sample buffer, pH 6.8, with 7 M urea and 1% 2-mercapto-ethanol, and separated as described in the electrophoresis section.

Antibody Preparation

Immunological cross reactivity between the enzymes was tested by using purified TL PE as an antigen to induce antibody formation in chickens. Animals were injected with 100 µg of TL PE and boosted in 2 weeks with an additional 100 µg. Antibodies were purified from egg yolk by the method of Jensenius et al. (1981). Yolk was separated from the white, diluted with 5 vol of distilled water and mixed until homogeneous. The egg yolk suspension was assayed by ELISA, as described in the next section, in order to select eggs with the maximum titer of anti-TL PE antibody. After adjusting to pH 7.0 with 0.1 N sodium hydroxide, the yolk suspensions with the highest titer were frozen at -20°C and thawed in a water bath (to coagulate lipids). Centrifugation at 5,000 x g for 20 min was necessary to remove aggregated lipids. Antibody was then precipitated with 50% ammonium sulfate. Precipitates were collected by centrifugation at 10,000 x g for 10 min, then dialyzed against 3 changes of 10 volumes of PBS-Tween buffer (0.02 M phosphate, 0.15 M sodium chloride, 0.5 g/L Tween and 0.1 g/L azide, pH 7.2).

ELISA Procedures

Antigens were diluted to the appropriate concentration (1-100 ng) into coating buffer, 0.1 M sodium carbonate, pH 9.6, and 100 µL added to each well of an Immulon plate (Fisher Scientific Company, Fair Lawn, NJ). After 2 h at room temperature (or overnight at 4°C), the Immulon plate was washed 4 times with PBS-Tween buffer. Antibody solution, 100 µL, diluted with PBS-Tween, was then added. After 1 h at room temperature (or overnight at 4°C), the plate was washed with PBS-Tween 4 times. Anti-chicken alkaline phosphatase conjugate (Sigma), was diluted 1/2000 with PBS-Tween, and 100 µL per well was added. After 1 h at room temperature, the plate was washed 4 times with substrate buffer (0.1 M sodium carbonate, 0.5 mM magnesium chloride, pH 9.4). Alkaline phosphatase substrate (p-nitrophenol acetate, Sigma), dissolved in substrate buffer (1mg/mL) was added (100 µL per well). The absorbance at 405 nm was read with an ELISA reader (Bio-Rad) when it reached 0.5-1.0 (within 0.5-2 h). If color development was too slow or too fast, antigen and/or antibody dilutions were adjusted accordingly. For blank determinations, either the antigen or primary antibody was omitted; the higher color intensity of the two is taken as the blank determination.

In competitive ELISA experiments, various amounts of competitor are mixed with the primary antibody solution prior to allowing it to interact with the bound antigen. Anti-TL PE antibody was used at 1/1000 and 1/2000 dilutions and 10 ng of TL PE was plated as antigen. Either TL PE or TS PE, 0-100 ng, were added as competitors to the anti-TL PE antibody.

Kinetics and Stability Studies

Initial Reaction Rates of TL and TS PE

The pH-activity profile was determined using Sunkist pectin as the substrate. Initial rates were measured titrimetrically between pH 5 to 8.5. Kinetic parameters were determined by measuring activity at substrate concentrations from 0.2-10 mg/mL for TL PE and 0.5-10 mg/mL for TS PE, using Sunkist pectin. Values for K_m and V_{max} were calculated from Lineweaver-Burk plots. The effect of DE of substrate on initial rate was examined by determining activity titrimetrically on the following pectin samples: HP Bulmer pectin (DE 20%, 35%, 46%, 68%), Sunkist (DE 70%), Kodak pectin (DE 62%).

Storage Studies

The stability of PEs was tested under technologically relevant storage conditions. To either 20 parts grapefruit juice concentrate (40° Brix), or to reconstituted single strength grapefruit juice (10° Brix), both at normal pH (3.3) and adjusted to pH 2.0 with hydrochloric acid, was added 1 part purified TL or TS PE. Sufficient enzyme was added to each juice sample to obtain an initial activity of 2.5 units/mL. Single strength samples were stored at 4°C, and 1 mL aliquots of concentrate samples were frozen and stored at -20°C. At time intervals, samples were taken and the residual activity was measured by titrimetric assay as described in the previous section.

The stability of TS PE to freeze-thaw trmatments was also examined. An aliquot of 0.5 mL of TS PE, initially about 80 units per mL, in 10 mM sodium phosphate, pH 7.0, was quickly frozen in a dry ice-ethanol bath, then thawed in a 40°C waterbath. Aliquots were assayed titrimetrically for total activity and thermostability (activity after 70°C for 5 min) after every fifth freeze-thaw. This process was repeated up to 40 freeze-thaw cycles.

Thermostability Studies

Residual activities of PEs after incubation for 5 min at various temperatures were determined. To tubes of identical dimensions containing 1 mL of single strength grapefruit juice (10° Brix) , pH 3.3, was added 0.05 mL of purified enzyme containing approximately 1 PE unit. After mixing, a thermocouple probe was placed in the tube and it was placed in a waterbath adjusted to a temperature at least 10°C higher than the incubation temperature to bring the temperature up rapidly. The tubes were then incubated for 5 min in a waterbath at the desired various temperatures. Residual activity was measured immediately after cooling on ice.

The rate of heat inactivation of TS PE in single strength grapefruit juice (10° Brix), pH 3.3, was determined at 75°C, 80°C, and 85°C. Pectinesterase, 5 units in 0.05 mL, was added to test tubes containing 1 mL reconstituted grapefruit concentrate of pH 3.3 and heat treated as described above for various times at the different temperatures. The log of per cent residual activity was plotted against heating time.

Stability of Pectinesterases to Proteolysis

Pectinesterases (10 μg) were incubated with 40 units of trypsin (Sigma) or pepsin (Sigma) at the respective pH optima (as provided by Sigma) of the proteases. Incubation with pepsin at room temperature was carried out in 100 mM acetic acid, pH 3, for up to 48 h. Incubation with trypsin was carried out at room temperature in 100 mM sodium phosphate buffer, pH 7, at room temperature for 48 h. Aliquots were assayed for activity at time intervals along with control samples incubated without protease under identical conditions.

Carbohydrate Composition and Function

Carbohydrate Assays

Total neutral sugar content of both purified enzymes was determined by the phenol-sulfuric acid method of Dubois et al. (1956). For analysis, 0.1-0.2 mg of TS PE or 0.5-1 mg of TL PE was dissolved in 1 mL distilled water. Phenol (25 µL, 80%) was added, followed by 2.5 mL of sulfuric acid. The tubes were allowed to stand for 10 min, then shaken and placed in a 30°C waterbath for 20 min before the absorbance at 490 nm was read. Glucose (from 10-50 µg) was used as a standard.

Hydrolyzates of purified TS PE or of O-linked oligosaccharides of TS PE (see Isolation and Analysis of O-linked oligosaccharides) were assayed for reducing sugar by the method of Waffenschmidt and Jaenicke (1987). A working reagent was made up fresh daily of equal volumes of reagent A and reagent B. Reagent A was a 5 mM solution of disodium 2,2'-bicinchoninate (Sigma) dissolved in 0.80 M sodium carbonate buffer, pH 10.1. Reagent B was 5 mM cupric sulfate pentahydrate (Fisher Scientific), and 12 mM serine (Sigma, S-4500) solution. To approximately 1 µg of carbohydrate in 100 µL distilled water in a screw cap vial was added 0.5 mL of working reagent. The vials were tightly capped and kept for 15 min at 100°C in an aluminum heating block. After cooling to room temperature for 20 min, samples were read at 560 nm in a Beckman DU 40 spectrophotometer. Glucose (1-10 nmol) was used as a standard.

Preparation of Alditol Acetates

Monosaccharides in both a total hydrolysate of purified TS PE and in a hydrolysate of O-linked oligosaccharide were converted to their respective alditol acetates and analyzed by gas chromatography/mass spectroscopy. Purified TS PE (100-200 µg) or O-linked oligosaccharide was hydrolyzed for 4 h in 2 N HCl at 100°C in a stoppered vial, then cooled to room temperature and neutralized with equimolar sodium hydroxide. The total hydrolysate of TS PE was then deionized by passage through an Amberlite MB3 column (1.4 x 5 cm). Fractions of 0.5 mL were collected and pooled based on reducing sugar content. Sugars were acetylated by the method of Blakeney et al. (1983). Sodium borohydride was dissolved in anhydrous dimethyl sulfoxide (20 mg/mL). Sugars were reduced at 40°C for 90 min by adding 1 mL of sodium borohydride solution to 0.1 mL of sample in 1 M ammonia. After reduction, excess sodium borohydride was destroyed by the addition of glacial acetic acid (0.1 mL). To the solution was added 1-methylimidazole, 0.2 mL, (Fluka, Buchs, Switzerland) followed by acetic anhydride (2 mL) with mixing. After 10 min at room temperature, water, (5 mL) was added to destroy excess acetic anhydride. After cooling, dichloromethane (1 mL) was added and the solution was mixed on a vortex mixer. After the phases had separated, the lower phase was removed with a Pasteur pipette and dried in a stream of N_2. After dissolving the residue in 50 µL of dichloromethane, 5-10 µL was subjected to analysis by gas chromatography/mass spectroscopy.

Gas Chromatography/Mass Spectroscopy

Gas chromatography was performed on a Finnegan 4500 GC/MS instrument. Derivatized sugars were separated on a DB5 GC column (30 M x 0.25 mm). A temperature program of 100-250°C at 5°C/min was chosen initially to ensure elution of all alditol acetates, including aminosugar derivatives, within a reasonable amount of time. The temperature program was later modified to 150-220°C at 2°C/min to optimize separation of the types of alditol acetates known to be present, based on the first analysis. Peaks were fragmented by electron impact at an electron energy of 70 eV. Chromatograms were constructed by monitoring the intensity of the fragment ion of m/z 115, a fragment ion which is diagnostic for alditol acetates. Alditol acetates were tentatively identified by comparison to the mass spectra of the following derivatized sugars: D-glucose (Sigma), D-galactose (Fisher Scientific), D-mannose (sigma), L-arabinose (Sigma), D-xylose (Sigma), D- ribose (Sigma), D-fucose (Sigma), and L-rhamnose (Sigma). GC/MS analysis of derivatized sugars was carried out by Dr.J. Toth at the Pesticide Research Laboratory, University of Florida.

Effect of Alkali on Serine and Threonine Residues

To test for the presence of *O*-glycosidic bonds in TS FE, 0.25 mL of enzyme (50 μg, 1 nmol) was mixed with 0.25 mL of 0.2 N sodium hydroxide at room temperature. The absorbance at 241 nm against a blank of 0.1 N sodium hydroxide was measured as a function of time by a Beckman DU8 spectrophotometer to determine the production of α-aminoacrylic acid and α-aminocrotonic acid by the β-elimination reaction (Neuberger et al., 1966). The number of *O*-glycosidic linkages per molecule of TS FE was estimated from the molar extinction coefficient at 241 nm (6050, given by Greenstein and Winitz, 1961) of the olefinic amino acids produced by the β-elimination reaction.

Isolation and Analysis of O-linked Oligosaccharide

In a separate experiment, TS FE was subjected to alkaline borohydride reduction by the method of Tanaka et al. (1964). Pure TS FE, 0.25 mL, containing approximately 50 μg protein or 7 μg carbohydrate (based on phenol sulfuric acid assay), was exhaustively dialyzed against 10 mM sodium phosphate buffer containing 1mM azide and added to 0.5 mL of a solution containing 0.45 M sodium borohydride and 0.15 N sodium hydroxide. After 48 h at 4°C in the dark, the solution was acidified to pH 5.3 with 1 N acetic acid to degrade excess borohydride. The solution was placed in a centricon ultrafilter with a 10 kD cut-off and centrifuged until 90% of the solution had filtered through the membrane. Thus isolated in the filtrate, the oligosaccharide was then hydrolyzed with 2 N hydrochloric acid for 4 h at 100°C in a screw cap vial and assayed for reducing equivalents as described in the carbohydrate assay section. The remaining portion of the filtrate (about 0.5 μg), was acetylated and analyzed by GC/MS as described in the GC/MS methods section.

Deglycosylation of TS PE

Samples of pure TS PE (containing 125 μg carbohydrate as determined by phenol-sulfuric acid assay) were subjected to periodate oxidation in 0.02 M sodium periodate in 0.05 M sodium acetate buffer, pH 5.0, for 2 h in the dark according to the technique of Pazur et al. (1963). After dialysis against deionized water at 4°C overnight, aliquots were assayed for protein, carbohydrate and enzyme activities. In a separate experiment, further degradation of the periodate-oxidized enzyme was carried out as follows: the pH was adjusted to 9.5 with sodium borohydride after periodate oxidation, then dialyzed again and adjusted to pH 1.0 with HCl (Smith and Unrau, 1959). Thermostable PE was also deglycosylated by the method of Sojar and Bahl (1987). This method consisted of incubating freeze-dried samples with trifluoromethanesulfonic acid (TFMSA), 150 μL/mg protein, at 0°C for 1 h followed by neutralization of the acid with aqueous pyridine, at -20°C. The neutralized reaction mixture was dialyzed at 4°C, then assayed for activity.

Enzymatic deglycosylation of TS PE was also attempted using N-glycosidase (Genzyme Corporation, Boston, MA) and a-mannosidase (Sigma, M7257) Enzyme concentrations and conditions used were as recommended by the manufacturer.

Effect of Deglycosylation on stability of TS PE

Thermostability of the periodate-oxidized enzyme was tested by incubation at 70°C for various periods of time in 10 mM phosphate buffer, pH 7.0 and the reaction was stopped by cooling on ice and assayed for PE activity. To evaluate storage stability, periodate-oxidized enzyme was spiked into single strength grapefruit juice, pH 3.3 and stored at 4°C for one week. Enzyme activity was tested at various time intervals.

RESULTS AND DISCUSSION

Purification

Purification of Thermolabile Pectinesterase (TL PE)

Ammonium sulfate fractionated crude grapefruit PE was further purified by ion exchange chromatography on CM-Sephadex. One activity peak was observed on CM-Sephadex column chromatography upon elution with 0.2 M sodium chloride as shown in Fig. 1. Fractions which eluted between 149 to 170 mL of this peak were pooled based on specific activity. When this material was applied to a column of Sephacryl-200, most of the PE activity eluted in a single large peak between 260 and 310 mL with minor activity eluting between 200 and 250 mL (Fig. 2). The fractions of the major peak eluting between 268-300 mL were pooled. The activity in the major peak was found to be inactivated completely by heating at 70°C for 5 min. The minor peak, however, was composed mostly of activity which was stable to this treatment. Based on Versteeg's (1979) results that PE forms in orange can be differentiated by heat stability, the major activity peak was designated thermolabile pectinesterase (TL PE), while the minor activity peak was

FIGURE 1

Purification of TL PE: Elution pattern from CM-Sephadex column chromatography of crude PE A preparation containing 13,240 units, derived from 300 g of pulp, was applied. Bound protein was eluted with 0.2 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.5.

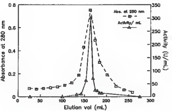
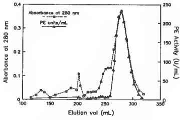

0.8
0.6
0.4
0.2
0
Absorbance at 280 nm
350
300
250
200
150
100
50
0
Activity (U/mL)
0
50
100
150
200
250
300
Elution vol (mL)
Abs. at 280 nm
Activity/ mL

FIGURE 2

Purification of TL PE: Isocratic elution pattern from Sephacryl-200 gel filtration column chromatography of CM-Sephadex fractions. A preparation containing 9,380 units, derived from 300 g of pulp, was applied. Proteins were eluted with 0.2 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.5.


Absorbance at 280 nm
PE units/mL
Absorbance at 280 nm
PE Activity (U/mL)
Elution vol (mL)
0
0.1
0.2
0.3
0.4
0
50
100
150
200
250
100
150
200
250
300
350

designated thermostable pectinesterase (TS PE). The specific activity of TL PE could not be increased by rechromatography or other chromatographic methods, such as HPLC gel filtration. After the 3rd purification step, TL PE appeared to be pure when analyzed by SDS-PAGE and HPLC gel filtration. The total yield of TL PE compared to activity in the crude extract was 19%. The purification of TL PE is summarized in Table 1. The yield of TS PE activity by this procedure was too low for further analysis (0.3%).

Purification of Thermostable Pectinesterase (TS PE)

Purification of thermostable PE was achieved by thermal inactivation followed by chromatographic methods. This form could not be separated from thermolabile form(s) by column chromatography alone due to the similarities in molecular properties and to difficulties in detection of minute amounts of TS activity in the presence of other active PEs. Removal of extraneous PE (mostly TL PE) activity was best accomplished by thermal inactivation followed by centrifugation. When the ammonium sulfate fractionated, crude PE solution was heated at 70°C for 5 min, the residual PE activity was generally found to be 5-10% of the original activity, therefore, it was necessary to start with a ten fold greater amount of pulp in the purification of TS PE. The best yields were obtained if the heating was carried out at pH 7.0 rather than at pH 7.5 or 8.0. The heat-treated crude PE solution was then applied

Table 1 Extraction and Purification of Thermolabile Pectinesterase [a]

Purification step	Activity [b] (units)	Protein [b] (mg)	Units/mg protein	Fold purif.	Yield (%)
Crude extract	8848 [c]	978 [d]	7	-----	100
Ammonium sulfate precipitation	4413	83	53	7.8	64
CM-Sephadex	3100	7.7	403	58	45
Sephacryl-200	1309 [e]	1.6 [f]	818	117	19

[a] The results of two purification procedures were averaged.
[b] Protein and activity values were calculated per 100 gm of grapefruit pulp.
[c] Replicates: 8163, 7533 units
[d] Replicates: 879, 1076 mg
[e] Replicates: 996, 1622 units
[f] Replicates: 1.03, 2.12 mg

to CM-Sephadex. One activity peak was observed on the CM-Sephadex elution profile (Fig. 3). From this step, a 50-fold purification of heat-treated crude FE was achieved. This material (elution volume 511 to 723 mL) was pooled and applied to a CM BioGel A column. The elution condition for the CM-BioGel A column was a linear sodium chloride gradient from 0 to 100 mM over 1000 mL. The increased resolution over that of the CM-Sephadex column served to separate a significant amount of bound contaminating protein. A single broad activity peak was observed in the elution profile shown in Fig. 4. Eluant fractions from 279 to 544 mL were pooled based on specific activity. This step provided a greater than 2-fold purification of the CM-Sephadex fractions. The concentrated fraction pool (10.8 mL) was applied to a G-75 gel filtration column. The elution pattern is shown in Fig. 5. A major activity peak of nearly uniform specific activity was observed (elution volume 188-203 mL). When analyzed by SDS-PAGE, the G-75 purified material migrated as a single sharp band. Rechromatography did not increase the specific activity, nor did HPLC gel filtration. The latter analysis showed a single peak of UV-absorbing material. The total yield of TS FE was 34%. The purification procedure of TS FE is summarized in Table 2.

FIGURE 3

Purification of TS PE: Gradient elution pattern from CM-Sephadex column chromatography of heat-treated crude PE. A preparation containing 6,510 units, derived from 3 kg, was applied. Protein was eluted with a linear 0-0.3 M sodium chloride gradient at pH 7.0.

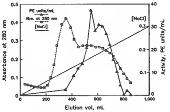

PE units/mL
Abs. at 280 nm
[NaCl]
[NaCl]
Absorbance at 280 nm
0.5
0.4
0.3
0.2
0.1
0
Activity, PE units/mL
40
30
20
10
0
0.3
0.2
0.1
0
200
400
600
800
1,000
Elution vol, mL

FIGURE 4

Purification of TS PE. Gradient elution pattern from CM-Biogel A column chromatography. A preparation containing 4800 units, derived from 3 kg, was applied. Bound protein was eluted by a linear 0-0.1 M sodium chloride gradient at pH 7.0

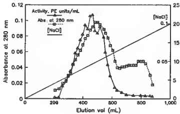

Activity, PE units/mL
Abs at 280 nm
[NaCl]
[NaCl]
0.1
0.05
Absorbance at 280 nm
0.12
0.1
0.08
0.06
0.04
0.02
0
Activity (U/mL)
25
20
15
10
5
0
Elution vol (mL)
0
200
400
600
800
1,000

FIGURE 5

Purification of TS PE. Isocratic elution pattern from G-75 gel filtration column chromatography. A preparation containing 4,020 units, derived from 3 kg, was applied. Proteins were eluted with 0.2 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.0.

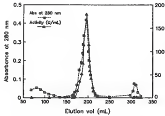

Abs at 280 nm
Activity (U/mL)
Absorbance at 280 nm
0.5
0.4
0.3
0.2
0.1
0
PE units/mL
200
150
100
50
0
50
100
150
200
250
300
350
Elution vol (mL)

Table 2. Extraction and Purification of Thermostable Pectinesterase [a]

Purification step	Activity [b] (units)	Protein [b] (mg)	Units/mg protein	Fold purity	Yield (%)
Crude extract	3690 [c]	1032 [d]	3.6	---	---
Ammonium sulfate precipitation	2664	134	20	---	---
Heat treatment	217 [e]	124 [f]	1.7	1	100
CM-Sephadex	160	2	60	46	74
CM-Bio-Gel A	139	0.65	214	122	64
Amicon conc.	134	0.38	353	202	62
G-75	74 [g]	0.14 [h]	529	302	34

[a] The results of two purification runs were averaged.
[b] Protein and activity values were calculated per 100 gm of grapefruit pulp.
[c] Replicates: 3330, 4053 units
[d] Replicates: 927, 1136 mg
[e] Replicates: 191, 242 units
[f] Replicates: 115, 134 mg
[g] Replicates: 70, 79 units
[h] Replicates: 0.130, 0.146 mg

Occurrence of Protease Activity in Crude and Partially Purified Preparations

Both crude extracts and partially purified PE were assayed for protease activity. At pH 4.5 after 24 h incubation, clearing zones were observed around the wells to which crude extract (7 ± 1 mm) and pepsin (12 ± 1 mm) and trypsin (10 ± 0 mm) controls were applied, but not around those containing partially purified PE or buffer even after 48 h. At pH 6.0, the wells containing crude extract (8.5 ± 0.5 mm) and trypsin (11.5 ± 0.5 mm) showed clearing zones after 24 h, while after 48 h a clearing zone appeared around the pepsin well (9.5 ± 0.5 mm). Neither the partially purified PE or buffer wells showed a clearing zone after 48 h of incubation. These results indicate that protease activity is present in the crude extracts, but is absent after ammonium sulfate fractionation and cation exchange chromatography. In order to minimize proteolytic breakdown of PE, crude extracts were fractionated with ammonium sulfate and cation exchange chromatography immediately after preparation.

Molecular Characterization

Determination of Molecular Weights

SDS-PAGE of TL PE in the presence of 2-mercaptoethanol showed a single band estimated at 37.3 ± 0.7 kD when stained with Coomassie blue/amido black (Fig. 6). A MW of 35.5 ± 0.8 kD was estimated for native TL PE by HPLC gel filtration on Superose-6 (Fig. 7). The agreement of these two methods indicates that the active enzyme is composed of a single polypeptide chain. SDS-PAGE of TS PE in the presence of 2-mercaptoethanol showed a single band estimated at 53.5 ± 1.3 kD when stained with Coomassie blue/amido black (Fig. 6). An estimate for the native MW of 51 ± 0.6 kD was obtained from HPLC gel filtration on Superose-6 (Fig. 7), thus indicating that the TS PE also is composed of a single polypeptide chain.

Ultraviolet Spectroscopy

The spectra of TL PE showed a maximum plateau between 274-281 nm and TS PE at 276-278 nm, with both spectra having minima at 250 nm. The ratio of absorbance at 280 to 250 nm was 1.55 for TL PE and 1.67 for TS PE. Based on protein determinations by the method of Smith et al. (1985), the extinction coefficient (1%, 1 cm) at 280 nm of TL PE and TS PE was estimated as 15.1 and 13.9, respectively. The available quantities of pure enzymes were insufficient to

FIGURE 6

Purified TS PE and TL PE on 12% SDS-PAGE mini-gel stained with Coomassie blue/amido black. Lane 1 contains 5 μg TS PE. Lane 2 contains 5 μg TL PE. Lane 3 contains the following standard proteins: phosphorylase b, 94 kD, bovine serum albumin, 67 kD; egg albumin, 43 kD; carbonic anhydrase, 29 kD; soybean trypsin inhibitor, 21 kD, α-lact-albumin, 14 kD. Electrophoresis was carried out as described in "MATERIALS AND METHODS".

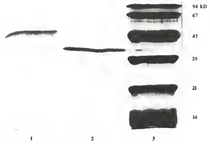
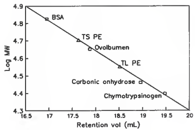

94 kD
67
43
29
21
14
1
2
3

FIGURE 7.

Determination of the native MW of TS PE and TL PE by gel filtration on HPLC Superose-6. Protein (100 μg) was eluted in 0.2 M sodium chloride in 10 mM sodium phosphate buffer at pH 7.0. Standards were bovine serum albumin (67kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and chymotrypsinogen (25 kD).


4.9
4.8
4.7
4.6
4.5
4.4
4.3
Log MW
16.5
17
17.5
18
18.5
19
19.5
20
Retention vol (mL)
BSA
TS PE
Ovalbumen
TL PE
Carbonic anhydrase
Chymotrypsinogen

determine these values more accurately by weighing. The observed differences in spectra suggest that these enzymes have different primary structures (Cantor and Schimmel, 1980).

Amino Acid Composition and N-Terminal Sequence Analysis

The amino acid composition of the grapefruit PEs are listed in Table 3. The enzymes were similar in content of certain amino acids. Both enzymes showed a high content of potential sites of glycosylation: Asx (Asp + Asn), 33 residues (12.3%) for TL PE and 54 (10.8%) for TS PE; Ser, 19 residues (7.2%) for TL PE and 32 (6.3%) for TS PE; Thr, 23 residues (8.5%) for TL PE and 36 residues (7.2%) for TS PE. The content of potentially acidic residues (Asx and Glx) was high: 20.2% and 19.6% for TL and TS PE, respectively. Since the pI of both enzymes is greater than 10, these amino acids would most probably be amidated. The content of basic residues, Lys and Arg together, is surprisingly low in both enzymes given their basic properties. The compositions of Navel orange and tomato PEs follow the same general pattern. In particular TL PE is very similar to Navel orange PEs in amino acid composition and average hydrophobicity, as well as thermostability properties (see Tables 4 and 5). The MW of TS PE was estimated based on amino acid composition and was similar to the SDS-PAGE and gel-filtration estimates. The estimate for TL PE was off by greater than 10% from the other methods.

Table 3. Amino Acid Composition of Purified Grapefruit Pectinesterases

Amino acid	TS PE		TL PE	
	mol %	mol/mol[a]	mol %	mol/mol[a]
Asp	10.8	46	12.3	35
Thr	7.2	31	8.5	24
Ser	6.3	27	7.2	21
Glu	8.8	38	7.9	23
Pro	4.0	17	2.8	8
Gly	10.4	45	10.5	30
Ala	9.7	42	12.5	36
Val	8.1	35	6.6	19
Met	0.7	3	0.7	2
Ile	4.7	20	4.4	13
Leu	7.2	31	5.7	16
Tyr	5.2	22	3.8	11
Phe	5.7	24	4.4	13
His	1.4	6	1.3	4
Lys	5.5	25	3.1	9
Arg	3.9	17	5.9	17
Cys	1.6	7	1.7	5
Trp	ND		ND	
Sum		436		286
Molecular weight		46,623		31,460
+ carbohydrate		54,340		32,102

[a] The calculations were based on met as the lowest common denominator. Ratios of the other amino acids to met were multiplied by 2 (TL PE) or 3 (TS PE).

Table 4 Amino Acid Composition of Purified Orange and Tomato Pectinesterases

Amino acid	Orange PE I[a] mol %	Orange PE I[a] mol/mol	Orange PE II[a] mol %	Orange PE II[a] mol/mol	Tomato PE[b] mol %	Tomato PE[b] mol/mol
Asp	11.06	32	9.69	28	10.3	31
Thr	8.62	25	7.94	23	8.3	25
Ser	6.37	19	7.28	21	5.0	15
Glu	6.77	20	7.34	21	8.6	26
Pro	3.69	11	3.57	10	4.0	12
Gly	8.87	26	11.05	32	9.9	30
Ala	11.55	34	10.80	31	10.6	32
Val	8.00	23	7.87	23	8.9	27
Met	1.55	5	1.56	5	2.3	7
Ile	5.33	16	5.31	15	5.3	16
Leu	5.31	15	4.64	14	3.3	18
Tyr	2.12	6	1.36	4	5.6	17
Phe	4.97	14	5.60	16	3.3	10
His	1.00	3	2.35	7	0.7	2
Lys	3.17	9	4.66	14	18.9	16
Arg	6.30	18	5.29	15	4.6	14
Cys	3.13	9	1.79	5	1.3	4
Trp	2.28	7	1.87	6	1.7	5
Sum		292		290		302
MW		32,120		31,900		33,220

[a] Versteeg, 1979

[b] Markovic and Jornvall, 1986

Table 5. Average Hydrophobicities of Pectinesterases and Selected Proteins

Source	Average Hydrophobicity (cal/res.)	Inactivation Temperature (°C)
Grapefruit		
TL PE	934	65
TS PE	1152	85
Orange [a]		
PE I	1030	70
PE II	1033	60
Tomato [b]	1022	
Elongation factor-Ts [c]		
T. thermophilus	1055	100
E coli	965	75

---

[a] Versteeg, 1979

[b] Markovic and Jornvall, 1986

[c] Nakamura et al., 1978

Thermostable PE had a notably higher concentration of hydrophobic amino acids (Phe, Tyr, Leu, Ile, Pro and Val) than did TL PE; on a mol% basis, TS PE contained approximately 35% hydrophobic residues to 28% for TL PE. Average hydrophobicity calculated for both enzymes by the method of Bigelow (1967), gave values of 1152 cal/residue for TS PE and 934 cal/residue for TL PE. The ratios of the volume of polar residues to the volume of non-polar residues, calculated by the method of Fisher (1964), were 0.553 for TS PE and 1.14 for TL PE. TS PE also is more hydrophobic than orange and tomato PEs (Tables 4 and 5). The reported correlation of increased protein hydrophobicity with greater thermostability (Mozhaev and Martinek, 1984) seems to hold up in the case of orange and grapefruit PEs (Table 5).

To determine the N-terminal sequence of TL PE, ten cycles of Edman degradation were carried out. No PTH-amino acids were detected in the column eluate, however. When the protein remaining in the sample after Edman degradation was hydrolyzed and the amino acids analyzed as described above, approximately 0.3 nmol of protein was detected. Enough protein was clearly present for sequence analysis, therefore, it seems likely that the N-terminus of TL PE is blocked.

Analysis of Cyanogen Bromide Cleavage Products

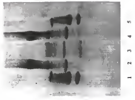
Cyanogen bromide selectively cleaves peptide bonds next to methionine (Gross and Witkop, 1962); therefore, the primary structure of the two forms of PE could be compared based on electrophoretic patterns of the cleavage products. SDS-PAGE of cyanogen bromide cleavage products are shown in Fig. 8. The cleavage products generated from 20 and 30 μg of TS PE were loaded onto lanes 2 and 4, respectively. The TS PE-derived fragments migrated as 3 bands. By comparing the widths of the bands with the widths of the standard protein bands, the quantity of protein in each band in lane 2 was estimated to be, from the top; 11, 1, and 4 μg (total=16 μg); the bands in lane 4 were estimated to contain 15, 3, and 5 μg (total=23 μg). The cleavage products generated from 50 μg of TL PE were loaded onto lane 3. The TL PE-derived fragments also migrated in 3 bands which, based on comparison of their widths with standards, contained 3 μg each. The TL PE fragments were apparently not well solubilized by the Laemmli buffer (with 7 M urea). The fact that no bands common to both enzymes can be seen in the electrophoretic patterns, suggests that these two forms of PE are separate gene products rather than single gene products with different post-translational modifications.

Estimation of Isoelectric Point

Both enzymes appear to be strongly basic, as both ran off at the cathode when isoelectrically focussed on a polyacrylamide gel of pH range 3-10 (data not shown). This result is not unexpected, since Versteeg (1979) found that

FIGURE 8

Analysis of cyanogen bromide cleavage products of TL and TS PE by SDS-PAGE. Bands were visualized with Coomassie brilliant blue/amido black. Lanes 1 and 5 contain standards: ribonuclease A (13.7 kD), a-lactalbumin (14.3 kD), and cytochrome C (12.5 kD). Lanes 2 and 4 contain approximately 16 and 23 μg of cleavage products of TS PE, respectively. Lane 3 contains approximately 9 μg of cleavage products of TL PE.


1
2
3
4
5

three PEs isolated from orange had isoelectric points greater than 10 (Table 6).

Antibody Preparation and Immunochemical Analysis

The titer of anti-TL PE chicken antibodies in the eggs was determined by ELISA (Fig. 9). Five eggs with the highest titer (day 18 though 24 after the initial injection) were pooled and used to analyze the cross-reactivity between the two enzymes by competitive ELISA. In the assay, both TL PE and TS PE were used as competitors for anti-TL PE antibodies against plated antigen, TL PE. As can be seen from Fig. 10, color intensity was reduced greatly (75-80% at both antibody dilutions), when 25 ng of TL PE was used as the competitor. When up to 100 ng of TL PE was added, little additional effect on intensity was seen. When TS PE was added as the competitor at 0-100 ng, the reduction in color intensity was nearly linear over the range. When 100 ng of TS PE was added, intensity dropped by 59% and 66% at antibody dilutions of 1/1000 and 1/2000, respectively. These results indicate that the PEs are partially cross-reactive and therefore, have shared structural components.

Table 6. Characteristics of Pectinesterases

Source	Molecular Weight	Isoelectric point	Spec. act. (u/mg)	K_m (mg/mL)	Optimal pH	Turnover no. (mol /mol·min)
Grapefruit						
TL PE	37,300[d]	≥ 10	776	0.274	7.0	26,354
TS PE	53,500[d]	≥ 10	540	1.02	7.8	30,621
Orange[a]						
PE I	36,200[d]	10	694	0.083	7.6	20,400
PE II	36,200[d]	≥ 11	762	0.0046	8.0	20,280
HMWPE	54,000[e]	10.2		0.041	8-8.5	
Orange[b]						
PE 1			222	0.25		
PE II			444	0.21		
Orange[c]			2200	2.3	8.0	
Tomato	23,700–35,500[e,f]	7-9.3[g]	704–1150[h,i,j]	2.4[h], 0.74[i]	8.0[h], 8.5[i]	39, 134[i]

[a] Versteeg et al., 1978; Versteeg, 1979
[b] Evans and McHale, 1978
[c] Manabe, 1973a
[d] estimated by SDS-PAGE
[e] estimated by gel filtration chromatography
[f] Pressey and Avants, 1972
[g] Delincee, 1976
[h] Nakagawa et al., 1970
[i] Lee and Macmillan, 1968
[j] Markovic, 1974

FIGURE 9

Titer of anti-TL PE chicken antibodies in eggs. Chickens were injected with 100 μg TL PE at day 0 and day 14. Eggs from day 18-24 were pooled for antibody purification. Further details are provided under "MATERIALS AND METHODS".

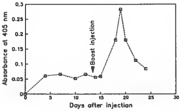
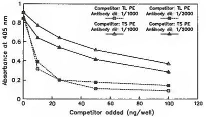

Absorbance at 405 nm
0
0.05
0.1
0.15
0.2
0.25
0.3
Boost injection
Days after injection
0
5
10
15
20
25
30

FIGURE 10

Analysis of antigenic properties of purified TS and TL PE by competitive ELISA TL PE, 10 ng, was plated and reacted with anti-TL PE antibody plus 0-100 ng/well of TL PE or TS PE as competitor. Further details are provided in "MATERIALS AND METHODS".


Competitor: TL PE
Antibody dil 1/1000
Competitor: TL PE
Antibody dil 1/2000
Competitor: TS PE
Antibody dil 1/1000
Competitor: TS PE
Antibody dil 1/2000
Absorbance at 405 nm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Competitor added (ng/well)

Kinetics and Stability

Kinetics

Kinetic parameters were determined from a double reciprocal plot of initial reaction rates measured at various concentrations of Sunkist pectin as a substrate (Fig. 11). The K_m and V_{max} for Sunkist pectin was 0.274 ± 0.040 mg/mL (1.02 ± 0.15 mM galacturonic acid) and 0.724 ± 0.056 µmol/min/µg for TL PE, and 1.02 ± 0.083 mg/mL (3.78 ± 0.31 mM galacturonic acid) and 0.59 ± 0.046 µmol/min/µg for TS PE. Turnover numbers on Sunkist pectin were 26,354 ± 2038 mol/mol·min for TL PE and 30,621 ± 2,387 mol/mol·min for TS PE. While the turnover numbers of the grapefruit PEs are similar, the K_m values are much higher than those of Navel orange (Table 6) This may partly reflect differences in substrate rather than in enzymes, however.

Optimal pH values of PEs on Sunkist pectin were around 7.0 for TL PE and between 7.5 and 8.0 for TS PE (Fig. 12). The very low activity of the enzymes at pH 5.0 on Sunkist pectin is undoubtedly due to the high DE of the substrate (70%). Versteeg (1979) showed that affinity for substrate was reduced greatly when the pH was decreased from 7.0 to 4.0. Since free carboxyl groups are necessary for enzyme-substrate complex formation (Solms and Deuel, 1955), the reduced affinity at low pH values can be explained by the decreased dissociation of the free carboxyl groups (the pK_a for pectin

FIGURE 11

Lineweaver-Burk plot of the activity of TS and TL PE at various concentrations of Sunkist pectin. Activity was measured by titrimetric assay. After adjusting the pH of 1% Sunkist pectin (25 mL) to pH 7.0 or 7.8 for TL or TS PE, respectively, enzyme solution (2 units/assay) was added. One PE unit was defined as 1 µmole of protons released per minute.

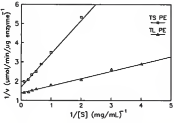
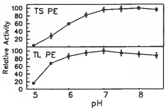

1/v (μmol/min/μg enzyme)$^{-1}$
1
2
3
4
5
6
1/[S] (mg/mL)$^{-1}$
0
1
2
3
4
5
TS PE
TL PE

FIGURE 12

The effect of pH on reaction rate of TS and TL PE on Sunkist pectin. Enzyme activity measured titrimetrically at all pHs as described for Fig. 11. Three units of enzyme activity (measured at optimal pH) was used per assay.


TS PE
TL PE
Relative Activity
100
80
60
40
20
0
5
6
7
8
pH

is about 4). On substrates with higher DE values, i.e., less carboxyl groups, the pH effect should be even more pronounced. The pH-activity relation at lower pH values shown here probably does not reflect the activities of these enzymes in citrus juice, since citrus juice would undoubtedly contain some pectin with lower DE values than that of the substrate used here.

The effect of DE on reaction rates is shown in Fig. 13 The measured activity of both enzymes increased similarly with decreasing DE. Versteeg (1979) reported a similar effect of DE on V_{max} of Navel orange PE I but not PE II. It can be safely assumed that the enzymes were at V_{max} under these conditions since the pectin concentrations used (10mg/ml) were much greater than the K_m determined on the least optimal substrate, Sunkist pectin (DE 70%). Therefore, DE affects both affinity (shown by Versteeg, 1979) and reaction rate.

Storage Stability

Enzyme activity of TL and TS PE was monitored under various storage conditions for up to 60 or 120 days (Figs. 14 and 15). Storage conditions were chosen for this study to simulate those normally encountered after processing. Storage stability in single strength grapefruit juice adjusted to pH 2 was included, because low pH inactivation of PE has been suggested as an alternative to pasteurization in citrus juice (Owusu-Yaw et al., 1988). TS PE was more stable than TL PE

FIGURE 13

Effect of degree of esterification on the reaction rate of TL and TS PE. Activities on pectins with various DE values were measured titrimetrically as described for Fig. 11. Two units of enzyme activity (measured on Sunkist pectin) were used per assay.

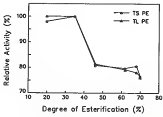

TS PE
TL PE
Relative Activity (%)
100
90
80
70
10
20
30
40
50
60
70
80
Degree of Esterification (%)

FIGURE 14

Storage stability of TL PE in grapefruit juice. Activity was measured titrimetrically after storage for various times in single strength grapefruit juice at 4°C (pH 2 and 3.3) and in frozen concentrate at -20°C.

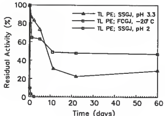
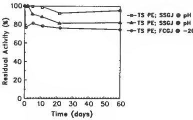

Residual Activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (days)
TL PE; SSGJ, pH 3.3
TL PE; FCGJ, −20° C
TL PE; SSGJ, pH 2

FIGURE 15

Storage stability of TS PE in grapefruit juice. Activity was measured titrimetrically after various times of storage in single strength grapefruit juice at 4°C (pH 2 and 3.3) and in frozen concentrate at -20°C.



Residual Activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (days)
TS PE; SSGJ @ pH 2
TS PE; SSGJ @ pH 3.3
TS PE; FCGJ @ -20

under all conditions tested. Particularly striking is the difference in stability in single strength juice at pH 2; TL PE was immediately inactivated while TS PE lost only 5% of activity after 60 days.

TS PE was also found to be extremely stable to repeated freezing and thawing. The initial activity was 348 ± 17 U/mL; after 40 freeze thaws, the activity was 347 ± 68 U/mL. The thermostability of the freeze-thawed material (checked by treating for 5 min at 70°C) was 330 ± 67 U/mL.

Thermal Stability

Thermostability of both enzymes was tested by heating for 5 min at various temperatures from 40-85°C at pH 7.0 in 10 mM phosphate buffer and measuring residual activity. A large difference in thermostability was observed between TL and TS PE (Fig. 16); TL PE and TS PE were inactivated completely at 65°C and 85°C, respectively. Thermal inactivation rates of TS PE at 75°C, 80°C, and 85°C in single strength grapefruit juice, pH 3.3, were 13.66, 2.77, and 0.3 min, respectively (Fig. 17). From this data, the change in temperature required to increase the inactivation rate 10-fold (z-value), was calculated to be 6°C. This data agrees well with other heat inactivation studies of grapefruit PE (Eagerman and Rouse, 1976). TS PE appears to be less thermostable than the counterpart in Navel orange (Table 6),

FIGURE 16

Heat stability of pectinesterases in grapefruit juice. Pectinesterases were heated at various temperatures in single strength grapefruit juice of pH 3.3 for 5 min. Residual activity was measured titrimetrically.

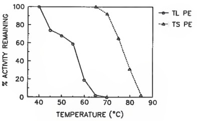
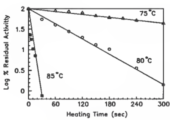

% ACTIVITY REMAINING
100
80
60
40
20
0
TEMPERATURE (°C)
40
50
60
70
80
90
TL PE
TS PE

FIGURE 17

Thermal inactivation rates of TS PE in grapefruit juice at 75°, 80° and 85°C. The activity of TS PE was measured after heating in single strength grapefruit juice of pH 3.3 for various times.


2
1.5
1
0.5
0
Log % Residual Activity
0
60
120
180
240
300
Heating Time (sec)
75°C
80°C
85°C

however, this could be due to the pH of grapefruit juice (pH 3.3) being lower than that of orange juice (pH 4.0).

Inactivation of Pectinesterases by Proteases

The susceptibility of both enzymes to inactivation by trypsin and pepsin was investigated. These two proteases have different bond specificity; pepsin cleaves at the carboxyl side of a wide variety of hydrophobic and acidic amino acids, while trypsin cleaves at the carboxyl side of Lys and Arg. Both PEs were fairly resistant to the action of trypsin; however, pepsin almost completely inactivated TL PE within 5 h, but had little effect on TS PE (Fig. 18). This result suggests that, unlike TL PE, the cleavage sites recognized by pepsin in TS PE are either absent or inaccessible. The inability of trypsin to significantly inactivate the enzymes suggests that the Lys and Arg residues are inaccessible at pH 7.0.

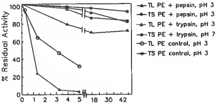
Composition and Function of Carbohydrate in Pectinesterases

Carbohydrate Content

Both enzymes after periodate oxidation were able to bind dansyl hydrazine, as visualized by fluorescence of bands with the expected R_fs on SDS-PAGE (data not shown). The bands of both a non-glycoprotein control (chymotrypsinogen) and un-

FIGURE 18

The effect of proteases on activity of pectinesterases. The pectinesterases were incubated with 40 units of either pepsin at pH 3.0 or trypsin at pH 7.0. Residual activity was measured titrimetrically as described for Fig. 11. No decrease in activity of either enzyme was observed in controls at pH 7.0 over 42 h (data not shown).


100
80
60
40
20
0
% Residual Activity
0
1
2
3
4
5
18
30
42
Time (hr)
TL PE + pepsin, pH 3
TS PE + pepsin, pH 3
TL PE + trypsin, pH 3
TS PE + trypsin, pH 7
TL PE control, pH 3
TS PE control, pH 3

oxidized PEs did not fluoresce, indicating specific binding of dansyl hydrazine to the carbohydrate moiety of the PEs.

In order to quantify the carbohydrate, the enzymes were assayed for total neutral carbohydrate. Total neutral carbohydrate content as estimated by the phenol-sulfuric acid assay using glucose as a standard gave the following results: TL PE, 2% ± 0.9%; TS PE, 14.2% ± 2.8%. If monosaccharides other than glucose are present, differences in color yields among the various sugars could introduce a source of error in this measurement.

Isolation of an *O*-linked oligosaccharide, followed by hydrolysis and assay of reducing equivalents gave a value of 20% ± 1.4% carbohydrate content for TS PE. An average MW for the reducing equivalents of 167 g/M, based on the composition determined by GC/MS, was used in the calculations. The fact that the total neutral sugar content is approximately equal to the amount of reducing sugar removed by alkaline borohydride reduction suggests that all the neutral sugars are alkali-labile and, therefore, *O*-linked. This rules out the possibility of large *N*-linked structures as they are stable under the mild alkaline conditions used.

Compositional and Partial Structural Analysis of the Carbohydrate Moiety of TS PE

The carbohydrate of TS PE was removed either by acid hydrolysis to release all sugars as monosaccharides or by reductive β-elimination to release *O*-linked oligosaccharides. In the gas chromatogram of the total hydrolysate (Fig. 19), four of the five peaks were identified by their electron-impact spectra as alditol acetates of a hexose (peak 4), two pentoses (peaks 2 and 3), and a deoxysugar (peak 1). Peak height ratios were 5.2:3:1:1. As accurate retention times were not obtained due to problems with the data collection system, the identity of the sugar acetates could not be determined conclusively. By comparison of the mass spectra to standard sugar acetates, the sugars were tentatively identified as fucose (peak 1), xylose (peak 2), arabinose (peak 3) and galactose (peak 4). Another sample was run under different elution conditions intended to enhance resolution of the sugars identified in the previous run. This time, two additional peaks (deoxysugar and hexose) were observed (Fig. 20). Peak height ratios were 5:3.5:2.7 for hexose:pentose:deoxysugar. However, the latter analysis may not be reliable as there was a limited amount of material for analysis.

Analysis of the products released by β-elimination as the alditol acetates is shown in Fig. 21. One hexose peak was observed (peak 2), however, the presence of other components at lower concentrations cannot be ruled out, since the amount of material available for analysis (0.5 µg) was limited. Also, other components may have been obscured by the internal standard (inositol, peak 1). By comparison with

FIGURE 19

Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides of the total hydrolysate of TS PE. About 5 μg of monosaccharides were analyzed. The alditol acetates were eluted with a temperature program of 100-250°C at 5°C/min. The chromatogram was constructed by scanning for the molecular ion of m/z 115. Peaks were identified as peracetylated: 1. deoxyhexitol, 2.pentitol, 3. pentitol, 4. hexitol.

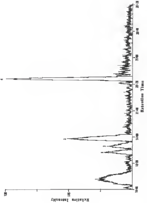

Relative Intensity
Retention Time
1
2
3
4

FIGURE 20

Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides from the total hydrolysate of TS PE. About 1 mg of monosaccharides were analyzed. The alditol acetates were eluted with a temperature program of 150-220°C at 2°C/min. The chromatogram was constructed by scanning for the molecular ion of m/z 115. The following peaks were identified as peracetylated: 3. deoxyhexitol, 4. deoxyhexitol, 5. pentitol, 6. pentitol, 7 myo-inositol (standard), 8 hexitol, 9. hexitol.

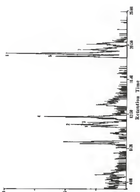
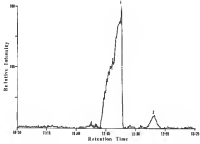

Relative Intensity
100
50
Retention Time
4:10
8:20
12:30
16:40
20:50
25:00
1
2
3
4
5
6
7

FIGURE 21

Electron impact gas chromatography/mass spectroscopy of alditol acetate derivatives of monosaccharides released from TS PE following reductive β-elimination and subsequent acid hydrolysis. About 0.5 µg of monosaccharides were analyzed. The alditol acetates were eluted with a temperature program of 150-220°C at 2°C/min. The chromatogram was constructed by scanning for the molecular ion of m/z 115. Peaks were identified as peracetylated 1. inositol, 2. hexitol.


1
2
100
115
Relative Intensity
10:50
11:15
11:40
12:05
12:30
12:55
13:20
Retention Time

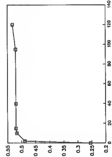
inositol the concentration of this component was calculated as 6.2% by weight of TS PE. However, quantitation by comparing the intensity of fragment ions from two different compounds is subject to error due to differences in response to ionization. This result, however, does indicate the presence of *O*-linked carbohydrate. Additional support for the *O*-linkage comes from the fact that aminohexoses, which are always present in *N*-glycosidic linkages, were not detected in TS PE, (Fig. 19).

Effect of alkali on serine and threonine residues

The cleavage of the *O*-glycosidic linkage between protein and carbohydrate moieties by a β-elimination reaction causes absorbance at 241 nm to increase as time proceeds due to formation of α-aminoacrylic acid and α-aminocrotonic acid from serine and threonine, respectively, (Neuberger et al., 1966). The increase in absorbance of TS PE, when placed in 0.1 N sodium hydroxide indicated that alkali-labile *O*-glycosidic bonds were involved in the linkage of protein to oligosaccharide (Fig. 22). *N*-linked carbohydrates are stable to mild alkali treatment. From the molar extinction coefficient of α-aminoacrylic acid at 240 nm (6050; Greenstein and Winitz, 1961), it was estimated that there are 23 *O*-glycosidic bonds per molecule of TS PE. Based on a carbohydrate content of 14.2% determined from the phenol-sulfuric acid assay, the average DP was calculated as 2.

FIGURE 22

β-elimination reaction of TS PE in alkali solution. A solution of purified TS PE was treated with alkali and the increase in absorbance at 241 nm was measured as described in "MATERIALS AND METHODS".


Absorbance at 241 nm
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
20
40
60
80
100
120
140
Time, min

Based on a carbohydrate content of 20%, determined as reducing equivalents in the hydrolyzed O-linked oligosaccharide, the average DP was calculated to be 2.8.

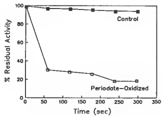
Deglycosylation of Thermostable Pectinesterase

Several researchers have reported that attached carbohydrate contributes to pH and thermal stability of glycoproteins (Chu et al , 1978; Hayashida and Yoshioka, 1979; Tashiro and Trevithick, 1976). Therefore it was hypothesized that the carbohydrate moiety contributed to the exceptional thermal stability of TS PE. Removal of the carbohydrate moiety of TS PE was attempted in order to substantiate this hypothesis

The use of periodate oxidation for this purpose was first suggested by Klappe (Pazur et al. 1963) and was later used by others (Pazur et al., 1970, Tashiro and Trevithick, 1977). Treatment of TS PE with periodate appeared to oxidize a portion of the carbohydrate residues while partially maintaining PE activity. During periodate oxidation of TS PE for 2 h, no change in amount of protein occurred, approximately 40% of the carbohydrate residues were oxidized as determined by the phenol-sulfuric assay. During this period, PE activity dropped to about 53% of the original activity. The periodate-oxidized enzyme was significantly less thermostable than native enzyme, when tested at 70°C in single strength grapefruit juice of pH 3.3 (Fig. 23). At 4°C

FIGURE 23

Heat stability of native and periodate-treated TS PE. TS PE was treated with periodate for 2 h as described in "MATERIALS AND METHODS". Enzyme activity was determined after various times at 70°C in single strength grapefruit juice (pH 3.3).


% Residual Activity
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Time (sec)
Control
Periodate—Oxidized

and pH 7.0, periodate-oxidized enzyme lost 75% of activity after 6 days (Fig. 24). It is possible that partial oxidation of Cys, Met and Trp may be contributing to the decrease in stability of periodate-oxidized TS PE. This has been shown to occur but at higher concentrations of periodate (0.08 N, Lee and Montgomery, 1961) than those used in this experiment (0.02 N).

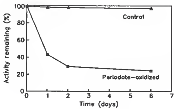
Further degradation of periodate-oxidized TS PE was carried out by reduction with sodium borohydride and mild acid hydrolysis (Smith and Unrau, 1959). This treatment, which should remove nearly all of the attached carbohydrate, resulted in total loss of activity and precipitation. Treatment of TS PE with TFMSA, a process which removes all carbohydrate from glycoproteins except N-linked hexosamines, also resulted in total loss of activity and precipitation of the enzyme. These results suggests that a certain amount of carbohydrate is necessary to maintain solubility and/or stability of TS PE.

Enzymatic treatment with N-glycosidase and a-mannosidase had no effect on activity, stability, or migration on SDS gels of TS PE. These results suggest that TS PE does not contain a significant amount of either N-glycosidic or a-mannosyl linkages.

Taken together, the results of the above experiments suggest that the carbohydrate moiety of TS PE makes a significant contribution to enzyme stability and solubility.

FIGURE 24

Stability of native and periodate-treated TS PE. TS PE was treated with periodate as described in "MATERIALS AND METHODS". Enzyme activity was determined after various times at 4°C in 10 mM sodium phosphate, pH 7.0.


Activity remaining (%)
100
80
60
40
20
0
Time (days)
0
1
2
3
4
5
6
7
Control
Periodate-oxidized

SUMMARY

The presence of two forms of PE in grapefruit pulp was established by this work. The forms differed in molecular weight, amino acid composition, cyanogen bromide cleavage patterns, carbohydrate content, pH-activity profile, turnover number, K_m, specific actvity, stability in grapefruit juice, stability to proteolysis and thermostability. These enzymes appear to have much in common with other PEs (Table 6). In particular, the molecular weight, isoelectric point, amino acid composition and thermostability properties of TL PE are very similar to PE I purified from Navel orange by Versteeg (1978), while TS PE shows similarities in molecular weight, isoelectric point and thermostability properties to the HMW pectinesterase isolated by Versteeg (1979).

This work represents the first time that a high MW, thermostable pectinesterase (TS PE) has been purified to homogeneity from citrus and also marks the first time that citrus PE has been shown to be glycosylated. Versteeg (1979) reported the presence of multiple forms of high MW PE in orange; however, in this study, no evidence was found for the existence of multiple forms of high MW, thermostable PE in grapefruit.

Amino acid composition served to distinguish TS and TL PEs (Table 3). In particular, TS PE has a 20% greater average hydrophobicity than TL PE and a 2-fold lower volume ratio of polar/non-polar residues. It was estimated that TL PE and TS PE contain 286 and 436 amino acids, respectively. The carbohydrate content of TL PE and TS PE were estimated to be 2% and 14.2%, respectively. No common bands were present in electrophoretic patterns of cyanogen bromide cleavage products of TS PE and TL PE. A limited amount of immunochemical cross-reactivity of the enzymes with anti-TL PE antibody was shown by competitive ELISA.

Comparison of the electrophoretic patterns of the cyanogen bromide cleaved enzymes suggests differences in primary structure, however, it is also possible that the differences in mobility of the cleavage products is due solely to variation in carbohydrate content. Differences in amino acid content, UV spectra and kinetic parameters, however, argue against the hypothesis that both enzymes are derived from a single gene product solely by post-translational glycosylation. Since immunochemical cross-reactivity indicates the presence of homologous structures, it is a possibility that limited proteolytic processing could account for the observed differences in these enzymes. Therefore, it is inconclusive from this work whether these enzymes originate from post-translational processing of a single gene product or from evolutionary divergence from a common precursor.

In the data presented here, TS PE was shown to be more thermostable and more resistant to acid pH and proteolysis, and has a longer storage life in grapefruit juice than TL PE. It is also extremely stable to freeze-thawing. These results support the findings of Versteeg (1979, 1980) and others that TS PE is the technologically significant form of PE in regard to citrus processing. Therefore, the focus should be on thermostable PE activity when exploring alternatives to thermal inactivation of citrus PE.

A positive correlation betwen protein hydrophobicity and thermostability has been well documented (Mozhaev and Martinek, 1984); therefore, increased hydrophobicity could account in part for the greater thermostability of TS PE.

TS PE was found to contain 7-fold more carbohydrate than TL PE. Thermostability analysis of periodate-oxidized TS PE suggests that the carbohydrate moiety contributes to the exceptional stability of TS PE. The increased carbohydrate content could also account for the greater resistance to proteolysis shown by TS PE. It has been shown that carbohydrate moieties provide protection against proteolysis (Coffey and de Duve, 1968; Tashiro and Trevithick, 1977).

An alternative processing strategy to thermal inactivation of TS PE is suggested by this work. Destabilization of TS PE by enzymatic removal of the carbohydrate moiety might be feasible. Additional knowledge of the carbohydrate structure would be helpful in selecting the best enzyme for this purpose.

The results of the immunochemical analysis indicates that a specific assay for TS PE may be a possibility. Since the enzymes, though similar, are not antigenically identical, monoclonal antibodies specific against TS PE should be obtainable. A specific ELISA for TS PE could then be developed which would be useful for quality control of citrus juice.

REFERENCES

Abo-El-Dahab, M.K. 1964. Production of pectic and cellulolytic enzymes by *Xanthomonas malvacearum*. Phytopath. 54: 597.

Ahern, T.J. and Klibanov, A.M. 1985. The mechanisms of irreversible enzyme thermoinactivation at 100°C. Science 228: 1280.

Arakji, O.A. and Jang, H.Y. 1969. Identification and characterization of the pectic enzymes of the McFarlin cranberry. J. Food Sci. 34: 340.

Argos, P., Rossman, M.G., Grau, U.M., Zuber, H., Frank, G., and Tratschin, J.D. 1979. Thermostability and protein structure. Biochemistry 18: 5698.

Aspinall, G.O. 1980. Chemistry of cell wall polysaccharides. In *The Biochemistry of Plants*, (J. Preiss, Ed.), Vol.3, p. 473. Academic Press, New York, London.

Bateman, D.F. 1963. Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extracts of *Rhizoctonia*-infected tissues of Bean. Phytopath. 53: 197.

Bateman, D.F. and Miller, R.L. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4: 119.

Baumann, G. and Gierschner, K. 1974. Research on technology of fruit juices produced from black currants. Ind. Obst Gemuseverw 59: 161.

Bell, T.A., Etchells, J.L., and Jones, I.D. 1951. Pectinesterase in the cucumber. Arch. Biochem. Biophys. 31: 431.

Biffen, J.H.F. and Williams, R.A.D. 1976. Purification and properties of malate dehydrogenase from *Thermus aquaticus*. In *Enzymes and Proteins from Thermophilic Microorganisms*, p. 157, (H. Zuber, Ed.). Birkhauser Verlag, Basel, Switzerland.

Bigelow, C.C. 1967. On the average hydrophobicity of proteins and the relation between it and protein structure. J. Theoret. Biol. 16: 187.

Binet, P. 1976. Succulence and pectinesterase activity in Aster tripolium. Physiol. Veg. 14: 293.

Bissett, O.W., Veldhuis, M.K., and Rushing, N.B. 1953. Effect of heat treatment temperature on the storage life of Valencia orange concentrates. Food Technol. 7: 258.

Blakeney, A.B., Harris, P.J., Henry, R.J., and Stone, B.A. 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydrate Research 113: 291.

Blumenkrantz, N., and Asboe-Hansen, G. 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484.

Brady, C.J. 1976. The pectinesterase of the pulp of banana fruit. Aust. J. Plant Physiol. 3: 163.

Brown, M.M. 1969. Pectin esters and the mechanical properties of plant cell walls. Ph.D. Thesis, Univ. Delaware.

Bruemmer, J.B., Roe, B., Bowen, E.R., and Buslig, B. 1976. Peroxidase reactions and orange juice quality. J. Food Sci. 41: 186.

Bryan, W.H. and Newcomb, E.H. 1954. Stimulation of pectinmethylesterase activity on cultured tobacco pith by indoleacetic acid. Physiol. Plant. 7: 290.

Cantor, C.R. and Schimmel, P.R. (1980). In Biophysical Chemistry. Part II, p. 374. W.H. Freeman and Co., San Francisco, CA

Castaldo D., Quagliuolo, L., Servillo, L., Balestrieri, C., and Giovane, A. 1989. Isolation and characterization of pectin methylesterase from apple fruit. J. Food Sci. 54: 653.

Chang, L.W.S., Morita, L.L., and Yamamoto, H.Y. 1965. Papaya pectinesterase inhibition by sucrose. J. Food Sci. 30: 218.

Chu, F.K., Trimble, R.B., and, Maley, F. 1978. The effect of carbohydrate depletion on the properties of yeast external invertase. J. Biol. Chem. 253: 8691.

Coffey, J.W. and de Duve, C. 1968. Digestive activity of lysosomes. J. Biol. Chem. 243: 3255.

Dahodwala, S., Humphrey, A., and Weibel, M. 1974. Pectic enzymes: Individual and concerted kinetic behavior of pectinesterase and pectinase. J. Food Sci. 39: 920.

Delecourt, R. 1972. Application of enzymes in the fruit juice industry. Bull. de l'Association des Anciens Eleves de l'Institut des Industries de Fermentation de Bruxelles 15: 82.

Delincee, H. 1976. Thin-layer isoelectric focusing of multiple forms of tomato pectinesterases. Phytochemistry 15: 903.

Deuel, H. 1947. On the glycoester of pectic acid. Helv. Chim. Acta 30: 1523.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350.

Eagerman, B.A. and Rouse, A.H. 1976. Heat inactivation temperature-time relationships for pectinesterase inactivation in citrus juices. J. Food Sci. 41: 1396.

Eckhardt, A.E., Hayes, C.E., and Goldstein, I.J. 1976. A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. Anal. Biochem. 73: 192.

Evans, R., and McHale, D. 1978. Multiple forms of pectinesterase in limes and oranges. Phytochemistry 17: 1073

Fisher, H.F. 1964. A limiting law relating the size and shape of protein molecules to their composition. Proc. Nat. Acad. Sci., Wash. 51, 1285.

Florida Crop and Livestock Reporting Service. 1985. Citrus Summary.

Fogarty, W.M. and Ward, O.P. 1974. Pectinases and pectic polysaccharides. In *Progress Industrial Microbiology*, D.J.D. Hockenhull (Ed.), p. 59-119. Churchill Livingstone, Edinburgh.

Fremy, E. 1840. Research on pectin and pectic acid. J. Pharm. Chim. 26: 368.

Gee, M., Reeve, R.M., McCready, R.M. 1959. Reaction of hydroxylamine with pectinic acids. Chemical studies and histochemical estimation of the degree of esterification of pectic substances in fruit. J. Agric. Food Chem. 7: 34.

Giulian, G.G., Shanahan, M.F., Graham, J.M., and Moss, R.L. 1985. Resolution of low molecular weight polypeptides in a non-urea sodium dodecyl sulfate slab polyacrylamide gel system. Fed. Proc. 44: 686.

Glasziou, K.T. 1957. The effect of 3-indolylacetic acid on the binding of pectin methylesterase to the cell walls of tobacco pith. Aust. J. Biol. Sci. 10: 337.

Glasziou, K.T. 1959. The localization and properties of pectin methylesterase of avena coleoptiles. Physiol. Plant. 12: 670

Glasziou, K.T. and Inglis, S.D. 1958. The effect of auxins on the binding of pectin methylesterase to cell walls. Aust. J. Biol. Sci. 11: 127.

Gonzalez, A.A., Canera, F.G., and Maguna, L.M. 1977. Enzymatic clarification of lemon juice. Ind. Aliment. Agric. 94: 283.

Greenstein, J. and Winitz M. 1961. In *Chemistry of the Amino Acids*. p. 859. Wiley, New York.

Gross, E. and Witkop, B. 1962. Nonenzymatic cleavage of peptide bonds: The methionine residues in bovine pancreatic ribonuclease. J. Biol. Chem. 237: 1856.

Hagerman, A.E and Austin P.J. 1986. Continuous spectrophotometric assay for plant pectin-methylesterase. J. Agric. Food Chem. 34: 444.

Hall, C.B. 1966. Inhibition of tomato pectinesterase by tannic acid. Nature 212: 717.

Hall, C.B. and Dennison, R.A. 1960. The relationship of firmness and pectinesterase activity of tomato fruits. Proc. Am. Soc. Hortic. Sci. 75: 629.

Hayashida, S. and Yoshioka, H. 1980. The role of carbohydrate moiety on thermostability of cellulases from *Humicola insolens* YH-8. Agric. Biol. Chem. 44: 481.

Hsu, C.P., Deshpande, S.N., and Desrosier, N.W. 1965. Role of pectin methylesterase in firmness of canned tomatoes. J. Food Sci. 30: 583.

Hultin, H.O. and Levine, A.S. 1963. On the occurrence of multiple molecular forms of pectinesterase. Arch.Biochem. Biophys. 101: 396.

Hultin H.O. and Levine, A.S. 1965. Pectin methyl esterase in the ripening banana. J. Food Sci. 30: 917.

Hultin, H.O., Sun, B., and Bulger, J. 1966. Pectin methyl esterases of the banana. Purification and properties. J. Food Sci. 31: 320.

Ishii, S., Kiho, K., Sugiyama, S., and Sugimoto, H. 1978. Low- methoxyl pectin prepared by pectinesterase from *Aspergillus japonicus*. Abstr. 5th Int. Congr. Food Sci. and Technol., 6s-14, Kyoto.

Ishii, S., Kiho, K., Sugiyama, S., and Sugimoto, H. 1979. Low- methoxyl pectin prepared by pectinesterase from *Aspergillus japonicus*. J. Food Sci. 44: 611.

Jansen, E.F., Jang, R., and Bonner J. 1960. Binding of enzymes to avena coleoptile cell walls. Plant Physiol. 35: 567

Jensenius, J.C., Andersen, I., Hau, J., Crone, M., and Koch, C. 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. J. Immunol. Meth. 46: 63.

Joslyn, M.A. and Pilnik, W. 1961. Enzymes and enzyme activity. In *The Orange: Its Biochemistry and Physiology*. W.B. Sinclair (Ed.), p. 373-435. Univ. of California Printing Dept.

Katchalski-Katzir, E.,and Freeman, A. 1982. Enzyme engineering reaching maturity. Trends in Biochemical Science. 7: 427.

Kauss, H. and Hassid, W.Z. 1967. Enzymic introduction of the methyl ester groups of pectin. J. Biol. Chem. 242: 3449.

Kauss, H., Swanson, A.L., Arnold, R., and Odzuck, W. 1969. Biosynthesis of pectic substances. Localization of enzymes and products in lipid-membrane complex. Biochim. Biophys. Acta 192: 55.

Kew, T.J. and Veldhuis, M.K. 1961. Cloud stabilization in citrus juice. U.S. Patent 2, 995, 448

Kohn, R., Furda, I., and Kopec, Z. 1968. Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. Collect. Czech. Chem. Commun. 33: 264.

Korner B., Zimmermann, G., and Berk, Z. 1980. Orange pectinesterase: Purification, properties and effect on cloud stability. J. Food Sci. 45. 1203.

Krop, J.J.P. 1974. The mechanism of cloud loss phenomena in orange juice. Agric. Res. Rep. 830, Pudoc, Wageningen.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Langcake, P., Bratt, P.M., and Drysdale, R.B. 1973. Pectinmethylesterase in Fusarium-infected susceptible tomato plants. Physiol. Plant Pathol. 3: 101.

Lee, M. and Macmillan, J.D. 1970. Mode of action of pectic enzymes. III. Site of initial action of tomato pectinesterase on highly esterified pectin. Biochemistry 9: 1930.

Lee, Y.S. and Wiley, R.C. 1970. Measurement and partial characterization of pectinesterase in apple fruits. J. Am. Soc. Hortic. Sci. 95: 465.

Leo, H.T. and Taylor, C.C. 1962. Method of making low sugar pectinic acid gels. U.S. Pat. 3,034,901.

Lineweaver, H. and Ballou, G.A. 1945. The effect of cations on the activity of alfalfa pectinesterase (pectase). Arch. Biochem. 6: 373.

MacDonnell, L.R., Jang, R., Jansen, E.F., and Lineweaver, H. 1950. The specificity of pectinesterases from several sources with some notes on the purification of orange pectinesterase. Arch. Biochem. 28: 260.

MacDonnell, L.R., Jansen, E.F., and Lineweaver, H. 1945. The properties of orange pectinesterase. Arch. Biochem. 6: 389.

Manabe, M. 1973a. Purification and properties of Citrus natsudaidai pectinesterase. Agric. Biol. Chem. 37: 1487.

Manabe, M. 1973a. Saponification of ester derivatives of pectic acid by Natsudaidai pectinesterase. J. Agric. Chem Soc. Japan, 47: 385.

Markovic, O. 1974. Tomato pectinesterase: Characterization of one of its multiple forms. Collect. Czech. Chem Commun. 39: 908.

Markovic, O. 1978. Pectinesterase from carrot (Daucus carrota). Experientia 43: 561.

Markovic, O., Heinrichova, K., and Lenkey, B. 1975. Pectolytic enzymes from banana. Collect. Czech. Chem. Commun. 40: 769.

Markovic, O. and Patocka, J. 1977. Action of iodine on the tomato pectinesterase. Experientia 33: 711.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262: 10035.

McColloch, R.J. and Kertesz, Z.I. 1947. Pectic enzymes. VIII. A comparison of fungal pectin methylesterase with that of higher plants, especially tomatoes. Arch. Biochem. 13: 217.

McCready, R.M. and Seegmiller, C.G. 1954. Action of pectic enzymes on oligogalacturonic acids and some of their derivatives. Arch. Biochem. Biophys. 50: 440.

Medina, P.V., Dostal, H.C., and Medina, M.T. 1981. Stabilization of pectin methylesterase of tomato fruit caused by ethylene at different temperatures. Rev. Ceres 28: 425.

Merkler, D.J., Farrington, G.K., and Wedler, F.C. 1981. Protein thermostability: correlations between calculated macroscopic parameters and growth temperature for closely related thermophilic and mesophilic bacilli. Int. J. Pept. Protein Res. 18: 430.

Mitek, M. and Drzazga, B. 1988. Interrelation between the effect of enzymic clarification of apple juices and the amount and quality of polyphenols. I. Inhibition of pectinolytic enzymes by polyphenols. Studies of model systems. Acta Aliment. Pol. 14: 175.

Moustacas, A., Nari, J., Diamantidis, G., Noat, G., Crasnier, M., Borel, M., and Ricard, J. 1986. Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. Part 2. The role of pectin methylesterase in the modulation of electrostatic effects in soybean cell walls. Eur. J. Biochem. 155: 191.

Mozhaev, V.V. and Martinek, K. 1984. Structure-stability relationships in proteins: new approaches to stabilizing enzymes. Enz. Microb. Technol. 6: 50.

Nakagawa, H., Sekiguchi, K., Ogura, N., and Takehana, H. 1971. Binding of tomato pectinesterase and β-fructofuranosidase to tomato cell wall. Agric. Biol.-Chem. 35: 301.

Nakagawa, H., Yanagawa, Y., and Takehana, H. 1970. Studies on the pectolytic enzymes. Part V. Some properties of the purified tomato pectinesterase. Agric. Biol. Chem. 34: 998.

Nakamura, S., Ohta, S., Arai, K., Arai, N., Oshima, T., and Kaziro, Y. 1978. Studies on polypeptide-chain -elongation factors from an extreme thermophile, Thermus thermophilus HB8. 3. Molecular properties. Eur. J. Biochem. 92: 533.

Nath, N. and Ranganna, S. 1977. Determination of thermal process for canned mandarin orange segments. J. Food Sci. Technol. 14: 113.

Neuberger, A., Gottschalk, A., and Marshall, R.D. 1966. In: Glycoproteins, A. Gottschalk (Ed.), p. 273, Vol. 5, Biochimica et Biophysica Acta Library. Elsevier Scientific Publishing Co., Amsterdam.

Neiderhiser, D.H., Plantner, J.J. and Carlson, D.M. 1971. The purification and properties of the glycoproteins of pig gallbladder bile. Arch. Biochem. Biophys. 145: 155.

Nieuwenhuis, C. 1978. Pectinesterase isozymes in citrus. M.Sc. Thesis, Dept. Food Sci., Agric. Univ., Wageningen.

Nozaki, Y. and Tanford, C. 1970. Solubility of amino acids, diglycine and triglycine in aqueous guanidine hydrochloride solutions. J. Biol. Chem. 240: 1646.

Owens, H.S., Lotzkar, H., Schultz, T.H. and Maclay, W.D. 1946. Shape and size of pectinic acid molecules deduced from viscometric measurements. J. Am. Chem. Soc. 68: 1628.

Owusu-Yaw, J., Marshall, M.R., Koburger, J.A., and Wei, C.I. 1988. Low pH inactivation of pectinesterase in single-strength orange juice. J. Food Sci. 53: 504.

Pazur, J.H., Kleppe, K. and Ball, E.M. 1963. The glycoprotein nature of some fungal carbohydrases. Arch. Biochem. Biophys. 103: 515.

Pazur, J.H., Knull, H.R., and Simpson, D.L. 1970. Glycoenzymes: A note on the role for the carbohydrate moieties. Biochem. Biophys. Res. Comm. 40: 110.

Pilnik, W. and Rombouts, F.M. 1978. Pectic enzymes. In: *Encyclopedia of Food Science*, M.S. Peterson and A.H. Johnson (Eds.), p. 604, Avi Publ. Company, Westport, Conn.

Pilnik, W., Rombouts, F.M., and Voragen, A.G.J. 1973. On the classification of pectin depolymerases. Activity on glycol esters of pectate as a new classification criterion. Chem. Mikrobiol. Technol. Lebensm. 2. 122.

Pozsar-Hajnal, K. and Polacsek-Racz, M. 1975. Determination of pectinmethylesterase, polygalacturonase, and pectic substances in some fruits and vegetables. Acta Aliment. 4: 271.

Prabhakaran, M. and Ponnuswamy, P.K.J. 1980. Spatial assignment of amino acid residues in globular proteins: An approach from information theory. J. Theoret. Biol. 87: 623.

Pressey, R. and Avants, J.K. 1972. Multiple forms of pectinesterase in tomatoes. Phytochem. 11: 3139.

Rexova-Benkova, L., and Markovic, O. 1976. Pectic enzymes. In: *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 33, p. 323, R.S. Tipton and D. Horton (Eds.). Academic Press, New York.

Rexova-Benkova, L., Markovic, O., and Foglietti, M.J. 1977. Separation of pectic enzymes from tomatoes by affinity chromatography on cross-linked pectate. Collect. Czech. Chem. Commun. 42: 1736.

Ricard, J. and Noat, G. 1986. Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. Part 1. A theory of the ionic control of a complex multi-enzyme system. Eur. J. Biochem. 155: 183.

Rombouts, F. M., Versteeg, C., Karman, B., and Pilnik, W. 1982. Pectinesterases in component parts of citrus fruits related to problems of cloud loss and gelation in citrus products. In: Use of Enzymes in Food Technology. P. Dupuy (Ed.), p. 483. Technique et Documentation Lavoisier, Paris.

House, A.H. and Atkins, C.D. 1952. Heat inactivation of pectinesterase in citrus juices. Food Technol. 7. 221.

Rouse, A.H. and Atkins, C.D. 1953. Maturity changes in pineapple oranges and their effect on processed frozen concentrate. Proc. Fl. State Hortic. Soc. 66: 268.

Scheraga, H.A. 1963. Intramolecular bonds in proteins. II. Non-covalent bonds. In: The Proteins, Vol. 1, 2nd ed., H. Neurath (Ed.), p. 477. Academic Press, New york.

Schultz, T.H., Lotzkar, H., Owens, H.S., and Maclay, W.D. 1945. Influence of method of de-esterification on the viscosity and acid behavior of pectinic acid solutions. J. Phys. Chem. 49: 554.

Segal, B., Segel, H., and Grager, W. 1966. Studies of the factors which influence the production of methyl alcohol when treated with pectolytic enzymes. Fluess. Obst. 33: 151.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H. Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76.

Smith, F. and Unrau, A.M. 1959. The presence of 1→6 linkages in laminaran. Chem. and Ind. 881.

Sojar, H.T., and Bahl, O.P. 1987. A chemical method for the deglycosylation of proteins. Arch. Biochem. Biophys. 259: 52.

Solms, J. and Deuel, H. 1955. On the mechanism of enzymatic saponification of pectic substances. Helv. Chim. Acta 38: 321.

Stellwagen, E. and Wilgus, H. 1978. Thermostability of proteins. In: Biochemistry of Thermophily, S.M. Friedman (Ed.), p. 223. Academic Press, London.

Tanner, H. 1970. On the production of low-methanol brandy. Schweiz. Z. Obst Weinbau 106. 626.

Tashiro, Y. and Trevithick, J.R. 1977. The role of carbohydrate in the glycoenzyme invertase of Neurospora crassa. Can. J. Biochem. 55: 249.

Termote, F., Rombouts, F.M., and Pilnik, W. 1977. Stabilization of cloud in pectinesterase active orange juice by pectic acid hydrolysates. J. Food Biochem. 1: 15.

Theron, T., De Villiers, O.T., and Schmidt, A.A. 1977. Isolation and purification of pectin methylesterase from Marvel tomatoes. Agrochemophysica 9: 93.

Versteeg, C. 1979. Pectinesterases from the orange: Their purification, general characteristics and juice cloud destabilizing properties. PhD thesis, Dept. of Food Science, Agricultural Univ., Wageningen, The Netherlands.

Versteeg, C., Rombouts, F.M., Spaansen, C.H., and Pilnik, W. 1980. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. J. Food Sci. Vol. 45: 969.

Versteeg, C., Rombouts, F.M., and Pilnik, W. 1978. Purification and some characteristics of two pectinesterase isoenzymes from orange. Lebensm. Wiss. Technol. 11: 267

Voragen, A.G.J., and Pilnik, W. 1970. Pectin research in the sixties. Dtsch. Lebensm. Rundsch. 66: 325.

Waffenschmidt, S. and Jaenicke, L. 1987. Assay of reducing sugars in the nanomole range with 2,2'-bicinchoninate. Anal. Biochem. 165: 337.

Weber, K. and Osborn, M. 1969. The reliability of molecular determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406.

Wedler, F.C., Hoffmann, F.M., Kenney, R., and Carfi, J. 1976. Maintenance of specificity, information and thermostability in thermophilic bacillus sp. glutamine synthetase. In: Enzymes and Proteins from Thermophilic Microorganisms, H. Zuber (Ed.), p. 187. Birkhauser Verlag, Basel.

Weurman, C. 1954. Pectase in Doyenne Boussoch pears and changes in the quantity of the enzyme during development. Acta Bot. Neerl. 3: 100.

Wicker, L., Vassallo, M.R., and Echeverria, E.J. 1988. Solubilization of cell wall bound thermostable pectinesterase from Valencia orange. J. Food Sci. 53: 1171.

Wiley, R.C. and Lee, Y.S. 1970. Modifying texture of processed apple slices. Food Technol. 24: 1168.

Wood, P. J. and Siddiqui, T.R. 1971. Determination of methanol and its application to measurement of pectin ester content and pectin methylesterase activity. Anal. Biochem. 39: 418.

Yoshihara, O., Matsuo, T. and Kaji, A. 1977. Purification and properties of acid pectinesterase from *Corticium rolfsii*. Agric. Biol. Chem. 41: 2335.

Zale, S.E. and Klibanov, A.M. 1984. Mechanisms of irreversible thermoinactivation of enzymes. Ann. New York Acad. Sci. 434: 20.

BIOGRAPHICAL SKETCH

Thomas A. Seymour was born on October 21, 1959, in Urbana, Illinois. He attended the University of Illinois at Urbana-Champaign, where he graduated with a Bachelor of Science degree in January, 1984. He received a Master of Science degree in bacteriology from the University of Wisconsin at Madison in August, 1985. He continued his graduate studies at the Department of Food Science and Human Nutrition at the University of Florida, Gainesville, where he was supported by a USDA National Needs Fellowship. He is expected to receive the degree of Doctor of Philosophy in May, 1990. He accepted a position as a postdoctoral associate in the Department of Immunology and Medical Microbiology at the University of Florida

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, a a dissertation for the degree of Doctor of Philosophy

James F. Preston, III
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, a a dissertation for the degree of Doctor of Philosophy.

Ben M. Dunn

Ben M. Dunn
Professor of Biochemistry and
Molecular Biology

This dissertation was submitted to the Graduate Facult of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

May, 1990

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School